



## **Bio-guided Fractionation of the Ethanolic Extract from Leaves of *Trema orientalis* Blume (Cannabaceae), a Presumed Antihypertensive Plant from Congo-Brazzaville**

**Bonazaba Milandou Longin Justin Clair<sup>1,2\*</sup>, Nkounkou Loumpangou Célestine<sup>1</sup>, Carreyre Hélène<sup>2</sup>, Bescond Jocelyn<sup>3</sup>, Vandebrouck Clarisse<sup>3</sup>, Etou Ossibi Arnaud Wilfrid<sup>4</sup>, Moutsambote Jean-Marie<sup>5</sup>, Ouamba Jean-Maurille<sup>1</sup>, Thibaudeau Sébastien<sup>2</sup> and Abena Ange Antoine<sup>4</sup>**

<sup>1</sup>Unit of Chemistry of Plant and of the Life, Faculty of Sciences and Technics, Marien Nguabi University, B.P. 69, Brazzaville, Congo.

<sup>2</sup>Superacid Group-Organic Synthesis Team, IC2MP-UMR CNRS 7582, University of Poitiers, 4 Rue Michel Brunet TSA 51106, 86073 Poitiers Cedex 9, France.

<sup>3</sup>STIM EA 7349 Laboratory, University of Poitiers, 1 Rue Michel Brunet TSA 51106, 86073 Poitiers Cedex 9, France.

<sup>4</sup>Biochimie and Pharmacology Laboratory, Faculty of Sciences of Health, Marien Nguabi University, B.P. 69, Brazzaville, Congo.

<sup>5</sup>Higher National School of Agronomy and Forestry, Marien Nguabi University, B.P. 69, Brazzaville, Congo.

### **Authors' contributions**

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

Hypertension is a risk factor for cardiovascular disease, which is currently a real public health problem. This disease affects about one billion people worldwide and is responsible for more than 70% of cardiovascular related deaths. Recently, the World Health Organization reported that of the hypertensive cases detected in Congo, only 7% were controlled. Today, there is no lifetime treatment and existing drugs are less accessible by the African population. To treat the disease, the Congolese population uses more the medicinal plants. However, the majority of compounds responsible for the biological activity of these plants are not known. In order to bring out Congolese plants with antihypertensive properties, we focus our interest on *Trema orientalis* Blume (Cannabaceae).

An ethanolic extract of the leaves of *Trema orientalis* was prepared after successive depletion of the organic solvents. Thereafter, a bio-guided fractionation on silica gel of the ethanol extract was carried out. Fractionation monitoring was done by TLC and the results of vasodilating activity measured. The fractions exhibiting the best biological activity allowed a second fractionation process to obtain five fractions which are characteristic of polyphenols, in particular flavonoids, and which exhibited good vasodilating activity on the isolated aorta of rats. Our future work will focus on the identification of these biologically active compounds.

**Keywords:** Bio-guide fractionation; ethanol extract; *Trema orientalis*; antihypertensive.

## 1. INTRODUCTION

Hypertension is a risk factor for cardiovascular diseases. Indeed, several studies point out the extremely harmful impact in the human populations of cardiovascular diseases and its common warning symptom: the increase of blood pressure. It currently represents a real public health problem as more than one billion people suffer from this disease worldwide [1-3].

In Europe, the global prevalence of hypertension was estimated at 31% in France in 2015, 16% in Switzerland in 2011 and 30% in England in 2006. However, in Northern America, the percentage of hypertension was 18% in the United States in 2014 and 23% in Canada in 2012 [4-6].

In African countries, hypertension is generally frequent with prevalence of 18.6% in Burkina Faso in 2014, 23% in Senegal in 2014, 29% in Guinea in 2006, 21% in Mali in 2007 and 12% in the Democratic Republic of Congo in 2006 [7-9].

In Congo, hypertension was responsible for 32 % in 2004 and 90% of cases of cardiovascular events in 2012 [10-12]. Recently, the results of a scientific study of the World Health Organization (WHO) revealed that of 27% of hypertensive cases detected in the Congo, 7% was controlled and 93% uncontrolled [13].

Today, the treatment of hypertension is one of the major challenges of public health. Despite the

efforts of the agencies, such as the WHO, the fight against this scourge is hampered by expensive clinical treatment and the price of drugs being out of reach of more than 80% of the population, especially the African population [13-15].

In order to cure diseases, most of the populations, especially the African population, use plants because they are more easily accessible, rich in natural compounds with therapeutic effects and less expensive than modern medicines [16].

In this context, it seems important to us to develop phytomedicines to fight effectively against arterial hypertension.

The use of plants in traditional African medicine to treat this disease encourages us to study compounds derived from plant biodiversity having antihypertensive activity.

As a result, we focus our interest on *Trema orientalis* Blume (Cannabaceae), a plant with a variety of biological activities, including arterial antihypertensive activity. This plant is used in traditional Congolese medicine to decrease blood pressure [17-26].

The aim of this work was to fractionate and evaluate the vasodilatory (antihypertensive) activity of the alcoholic extract and its derived fractions from *Trema orientalis* Blume leaves.

## 2. MATERIALS AND METHODS

### 2.1 Plant Material

*Trema orientalis* Blume leaves were harvested in Mayitoukou, a village located at south-west from Brazzaville, the capital city of Republic of Congo. The identification of the plant was made by a botanist of the Center of Studies of Plant Resources of Brazzaville. A specimen was recorded with the national herbarium (Sita, N445).

These leaves were dried out of the sun and at room temperature for three weeks and then crushed. The resulting powder was stored in translucent glass vials in the laboratory.



Fig. 1. *Trema orientalis* Blume

### 2.2 Biological Material (Animal)

The male Wistar rats (January labs, Genest Saint Isle, France) weighing 250 to 500 g and aged 4 to 6 months old were used to perform biological tests. The animals were kept under constant conditions of temperature (22-23°C) and humidity (50-60%) with regular cycles of 12 hours of day/12 hours of dark (PREBIOS, University of Poitiers). They also had free access to water and food. The care provided to the rats was in accordance with the directives of the European Community on the use of laboratory animals (L358-86/609EEC).

### 2.3 Methods

#### 2.3.1 Ethanolic extract preparation: Soxhlet extraction by increasing polarity solvents

The ethanolic extract was obtained after successive depletion of the plant material with

hexane, chloroform, ethyl acetate according to their increasing polarity order.

An amount of 100 g of dried powder of plant leaves was introduced into a cartridge placed in the soxhlet surmounted by a condenser and carried by a 1000 mL flask containing 500 mL of extraction solvent. A series of siphoning made it possible to carry out the extraction until exhaustion of by the used solvent. Extraction of the plant material was stopped when the extraction solvent became increasingly clear.

The first extraction was done with hexane to obtain the hexanic extract after evaporation of the solvent in a rotavapor at 45°C. The residue solid was dried and taken under the same conditions, in increasing order of their polarity, with chloroform, ethyl acetate and ethanol to obtain respectively chloroformic, ethyl acetate and ethanolic extracts.

This operation was repeated several times to obtain a large mass of each extract. The dry extracts obtained after evaporation of the solvent were then stored at room temperature, protected from light until they were used.

#### 2.3.2 Bio-guided open-column fractionation of the ethanolic extract and its fractions

##### 2.3.2.1 Bio-guided fractionation of the ethanolic extract

The ethanolic extract was then tested on isolated rat aorta rings and exhibited vasodilator activity.

5 g of ethanolic extract were deposited on a column of silica gel (Kieseigel 60 type) prepared in hexane. The elution was carried out by a polarity gradient of the hexane/ethyl acetate system (100/00, 70/30, 50/50, 80/20, 00/100) followed by another mixture system ethyl acetate/ethanol (90/10, 80/20, 60/40).

Fractionation monitoring was done by TLC. Plates were visualized under UV light (365 nm) after exposure to Neu and visible after revelation with anisaldehyde followed by heating at 100°C for 5 min [27]. The obtained fractions were grouped according to their chromatographic profile and a total of nine fractions F1 to F9 were obtained after evaporation of the solvent on a rotary evaporator at 40°C.

This operation was carried out four times in order to collect 20 g of the ethanolic extract and to increase the mass of the fractions.

Subsequently, the nine obtained fractions were tested on the rings of aorta isolated from rat to verify the presence of a vasodilator effect.

#### 2.3.2.2 Fractionation of the most active fraction (F9) on vasodilator activity

The fraction F9 obtained in the ethyl acetate/ethanol mixture (80/20 and 60/40) was then refractioned on silica gel in an open column. Two binary and tertiary systems of elution solvents were used successively following a gradient of increasing polarity. These systems were respectively petroleum ether/ethyl acetate (99/1, 0/100) and ethyl acetate/methanol/formic acid (90/8/2, 85/11/4, 82/14/4, 80/15/5, 77/16/7, 60/40/0).

The fractionation was followed by TLC; the plates were visualized under UV light at 365 nm after revelation with Neu [27]. The collected subfractions were grouped according to their thin layer chromatographic profile and a total of ten subfractions (F9-1 to F9-10) were obtained. Subfraction F9-1 (colorless liquid) showed a single spot on TLC.

Six of the ten subfractions were selected for their chromatographic profile and were bioassayed.

#### 2.3.2.3 Fractionation of the most active subfraction F9-5 on vasodilator activity

Subfraction F9-5 obtained in the ethyl acetate/methanol/formic acid system (82/14/4) was refractioned in a ternary isocratic system of ethyl acetate/ethanol/water 95/10/5.

The subfractions obtained were grouped according to their thin layer chromatographic profile and a total of three subfractions (yellow solid) noted FA, FB, FC were obtained after evaporation of the solvent on a rotary evaporator at 40°C.

Each of the subfractions FB and FC presented a single spot on TLC. They were then tested on the biological model of vascular reactivity.

#### 2.3.2.4 Fractionation of the most active subfraction F9-6 on the vasodilator

Subfraction F9-6, intermediate between subfractions F9-5 and F9-7 obtained in the system ethyl acetate/methanol/formic acid (82/14/4) was not tested on the biological model. It was fractionated on silica gel with the same isocratic solvent system of ethyl acetate/ethanol/

water (76/19/05) giving three subfractions (FD, FE and FF) of yellow powder after grouping according to their chromatographic profile.

The subfraction FE thus collected in a mixture of ethyl acetate/ethanol/water 76/19/5 was refractioned in the same elution system with polarities 85/10/5 and 77/17/6 to give the yellow powder subfractions noted FE-1, FE-2 and FE-3.

Each of the subfractions FE-1 and FE-3 obtained after evaporation of the solvent on a rotary evaporator at 40°C showed a single spot on TLC. Subfraction FE-3 was tested in vascular reactivity.

#### 2.3.2.5 Fractionation of subfraction FG

Subfraction FG was obtained by pooling two fractions F9-7 and FF collected respectively in ethyl acetate/methanol/formic acid (82/14/4) and ethyl acetate/ethanol/water (76/19/5). This fraction was refractioned by elution using a ternary mixture of solvent (ethyl acetate/ethanol/water) following the 85/10/5 and 78/17/5 gradients to give the fractions FG-1, FG-2 and FG-3. Subfraction FG-3 (brown soft solid) showed a single spot on TLC. The mass of the subfractions was obtained after evaporation of the solvent on a rotary evaporator at 40°C. Subfraction FG-3 was biologically tested on isolated rat aorta rings.

## 2.4 Antihypertensive Tests (Vasodilator) on the Ethanolic Extract and Its Fractions

### 2.4.1 Collection and assembly of aorta segment of a rat

The rat was anesthetized by intraperitoneal injection of pentobarbital (20 mg/kg). An abdominal and thoracic incision to expose the thoracic aorta, located along the spinal column behind the heart, was performed. The thoracic portion of the aorta is cut and placed in a petri dish containing physiological Krebs solution at pH 7.4 and at 37°C. After removing the adherent tissue, the thoracic aorta segment was cut into rings about 3 mm in length. Each aortic ring was suspended between two stainless steel hooks in a 5 mL insulated organ chamber, bubbled with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>, dipping into a thermostatically controlled water bath containing distilled water at 37°C. One of the hooks was attached to the tank while the other was connected to an IT1-25 force displacement

transducer (Emka Technologies). A basal tension of 2 g was applied to the aorta allowed to balance in all experiments for 1 h. During this time, the aorta was rinsed three times with Krebs solution [28].

#### 2.4.2 Evaluation of the vasodilator effects of ethanolic extract and its fractions

Norepinephrine ( $10^{-6}$  M) was added to the organ cup to cause sustained contracture of the aorta. When the contracture plateau is reached, the extracts or fractions of the leaves of the plant at 1 mg/mL were added to the organ tank. The concentration-cumulative-response relationship for the relaxing effect of these extracts or fractions was determined in the aortic rings following stable contraction [28,29]. The data from these experiments allowed further fractionation and validation of this biological activity.

### 3. RESULTS AND DISCUSSION

#### 3.1 Yields of the Different Obtained Extracts

The masses (the yields) of the successive extraction for 100 g of the powder of the plant material are 3.61 mg (3,61%) for hexane extract, 1.60 mg (1.60%) for chloroform extract, 0.95 mg (0.95%) for ethyl acetate extract and 4.95 mg (4.95%) for ethanolic extract.

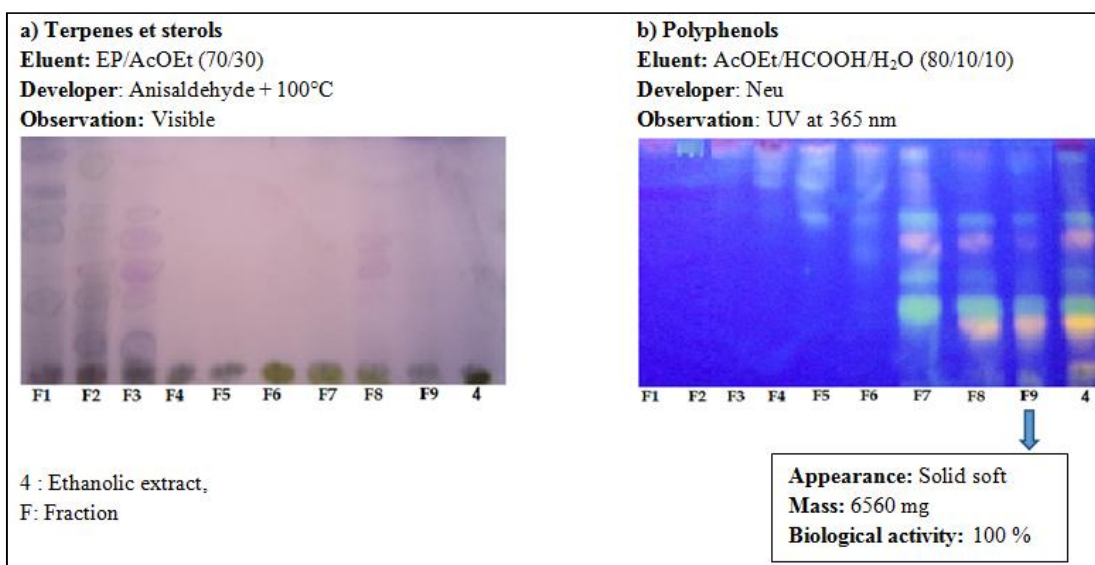
#### 3.2 Bio-guided Open-column Fractionation of the Ethanolic Extract and Its Fractions

##### 3.2.1 Fractionation of the ethanolic extract

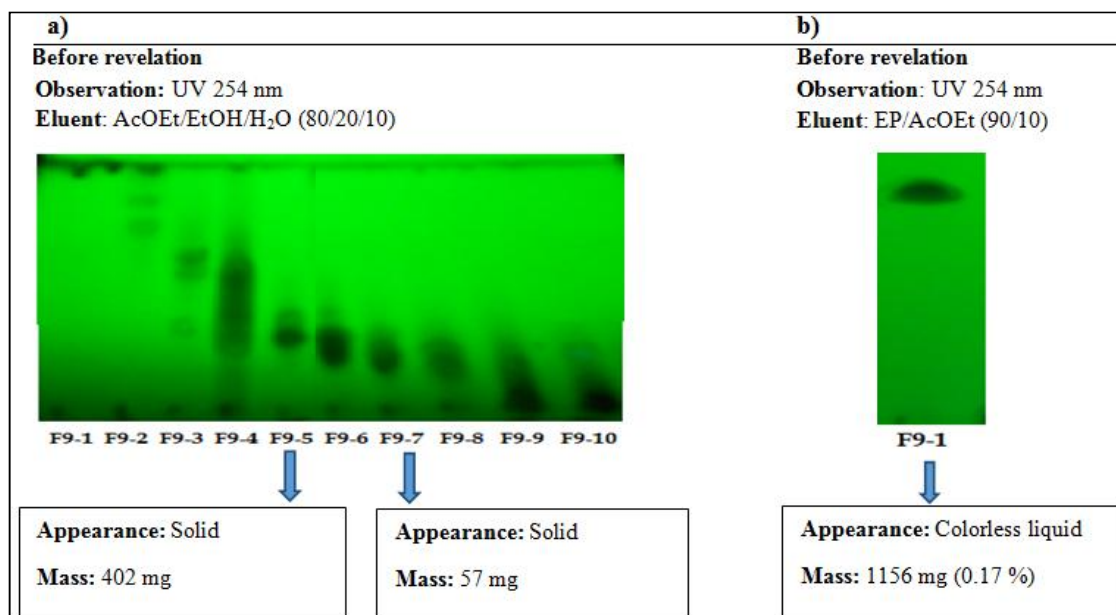
The fractionation steps on the column chromatography covered with this extract were guided by the thin layer chromatographic profile as a function of the major chemical families in this plant organ and by the results of the vasodilator (antihypertensive) activity.

Fractionation of this extract in the binary elution systems (petroleum ether/ethylacetate and ethylacetate/ethanol) gave nine fractions which were obtained and grouped according to their chromatographic profiles (Fig. 2). The chromatogram of the nine fractions, revealed by anisaldehyde, observed in the visible then heating at  $110^{\circ}\text{C}$ , indicated the presence of violet and brown spots characteristic of terpenes and steroids (Chromatogram no 1a). The observation of this chromatogram with UV light at 365 nm, after revelation with Neu, showed the presence of blue, yellow-orange and orange spots and fluorescence spots attributable to polyphenols, in particular to phenolic acids and flavonoids (Chromatogram no 1b) [27].

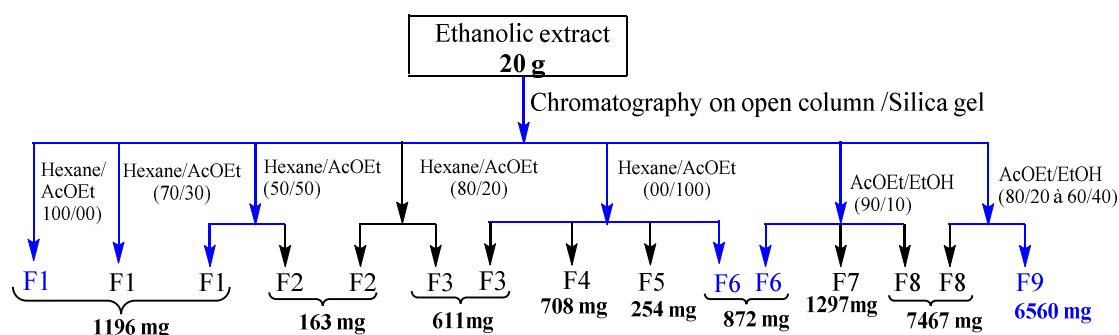
However, there is a dominance of terpenes and sterols in fractions F2 to F3 and flavonoids in fractions F7 to F9 of this extract.



Chromatogram No. 1. TLC of terpenes/sterols and polyphenols of nine fractions



**Chromatogram No. 2. TLC polyphenols of 10 subfractions**



**Fig. 2. Fractionation scheme of the ethanolic extract**

Indeed, previous work on the leaves of this Congolese plant species has revealed the presence of terpenes, sterols and polyphenols (flavonoids, tannins, coumarin) [20]. These results are consistent with those found in Nigerian, Ivorian, Cameroonian, Benin, Indian, South African and Taiwanese literature [21,23-26,30-35].

The biological test of these nine fractions showed a vasodilator activity whose best activity (100%) was observed with the fraction F9 (dark brown) (Fig. 3).

### 3.2.2 Fractionation of fraction F9

In view of the interesting biological activity of the F9 fraction obtained in the ethyl acetate/ethanol (80/20) elution system, we decided to fractionate

it. As a result, the fraction F9 underwent open-column fractionation with the binary and tertiary eluate systems to give 10 subfractions denoted F9-1 to F9-10 (Fig. 4) according to the chromatographic profile revealed in the Neu and observation at UV 365 and 254 nm. The chromatographic profiles observed underline at UV 254 nm the presence of the more prominent polar compounds of the subfractions F9-3, F9-5, F9-6, F9-7, F9-9 and F9-10 that can be attributed to polyphenols (chromatogram No. 2) [16,20,27].

Subfraction F9-1 with a colorless oily appearance and a single spot on TLC at 254 nm UV was very large (1156 mg).

The vascular reactivity test performed on these six selected subfractions was positive with a



better activity concentration in the F9-1 and F9-5 subfractions (Fig. 5).

### 3.2.3 Bioguided fractionation of fraction F9-5

Depending on the vasodilator activity of the fraction F9-5 obtained in the form of the yellow

powder and in the ethyl acetate/methanol/formic acid elution system (82/14/4), this fraction was fractionated on silica gel using a single solvent gradient of ethyl acetate/ethanol/water (AcOEt/EtOH/H<sub>2</sub>O: 85/10/5). Three subfractions (FA, FB and FC) of yellow powder were obtained (Fig. 6).

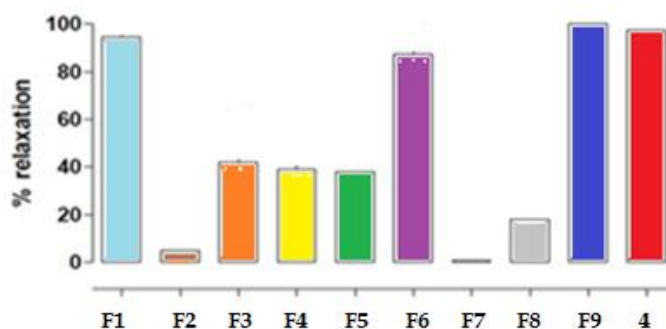


Fig. 3. Results of vasodilator activity of the ethanolic extract and its fractions tested at 1 mg/mL

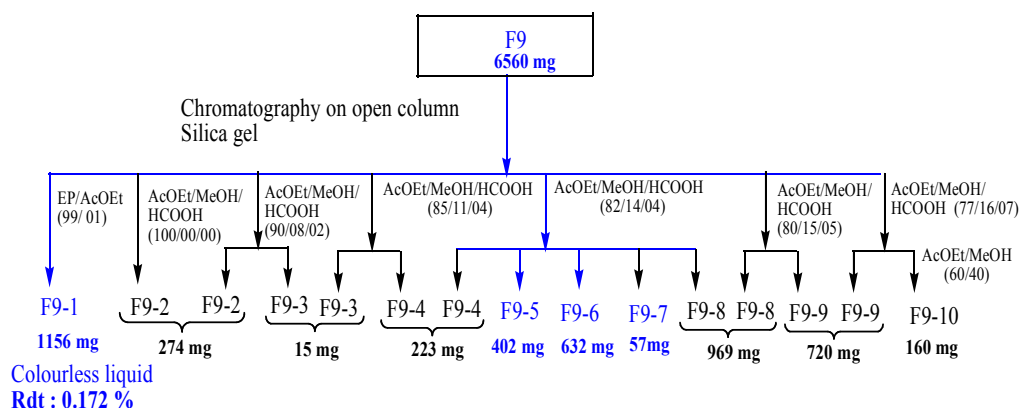


Fig. 4. Fractionation scheme of fraction F9

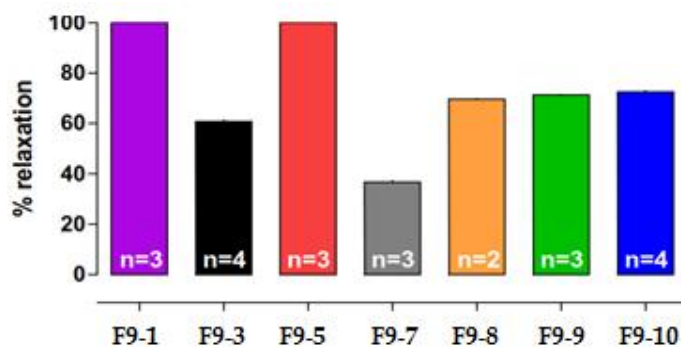


Fig. 5. Result of vasodilator tests of F9 subfractions tested at 1 mg/mL, n: Repetition of test numbers

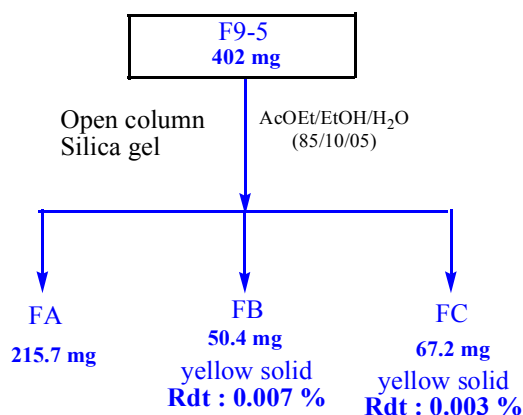


Fig. 6. Fractionation scheme of fractions F9-5

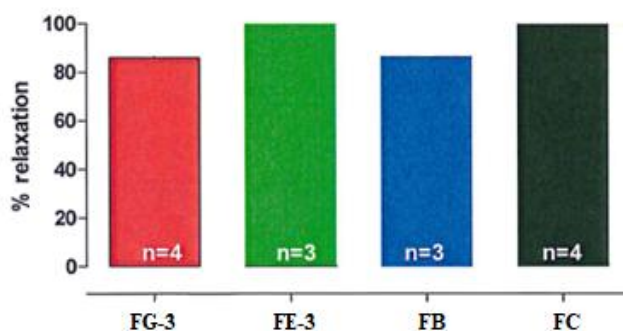


Fig. 7. Result of the vasodilator tests of subfractions FG-3, FE-3, FB and FC tested at 1 mg/mL.  
n: Repetition of test numbers

Subfractions FB and FC obtained and observed at UV 254 nm before revelation and UV 365 nm after revelation at Neu each showed a single spot of 0.38 frontal ratio in a solvent system ethyl acetate/formic acid/water (AcOEt/HCOOH/H<sub>2</sub>O: 80/10/10) (chromatogram No. 3).

These two subfractions exhibited an interesting vasodilator activity (Fig. 7).

### 3.2.4 Fractionation of fraction F9-6

Subfraction F9-6 obtained in the solvent system ethyl acetate/methanol/formic acid (82/14/4), was not tested for biological activity. Given its chromatographic profile which is intermediate between subfractions F9-5 and F9-7, it was fractionated with the same solvent system as F9-5 but of different polarity (AcOEt/EtOH/H<sub>2</sub>O: 76/19/05). Three subfractions (FD, FE and FF) of yellow powder were obtained after grouping according to their chromatographic profile.

The resulting FE was then fractionated and also resulted in three yellow powder subfractions noted FE-1, FE-2 and FE-3 (Fig. 8).

Subfractions FE-1 showed the same chromatographic profile as subfraction FC. FE-3, counted, showed a single green UV at 356 nm after revelation at Neu and a frontal ratio of 0.29 (chromatogram No. 4). This fraction showed good vasodilator activity (Fig. 7).

### 3.2.5 Fractionation of fraction FG

The subfractions FF and F9-7, having the same chromatographic profile, were grouped together and denoted FG. This fraction was then fractionated in the solvent system AcOEt/EtOH/H<sub>2</sub>O (85/10/05 and 78/17/05) and gave three subfractions FG-1, FG-2 and FG-3 (Fig. 9).

In contrast to the subfractions obtained previously, the brown fraction FG-3 obtained in



the same eluent AcOEt/EtOH/H<sub>2</sub>O (78/17/05) exhibits, on TLC in AcOEt/EtOH/H<sub>2</sub>O (80/20/10), a yellow-orange spot at UV 356 nm after exposure to Neu and the same frontal ratio (R<sub>f</sub> = 0.29) than the FE-3 (chromatograms Nos. 4 and 5). The FG-3 bioassay was found to be positive (Fig. 7).

These results confirm the work done by several authors who have shown the presence of terpenoid and polyphenols in the polar extracts obtained from the leaves of the plant [21,23-26,30-35].

### 3.3 Evaluation of the Vasodilator Effect of the Ethanolic Extract and Its Fractions Derived from *Trema orientalis* Blume

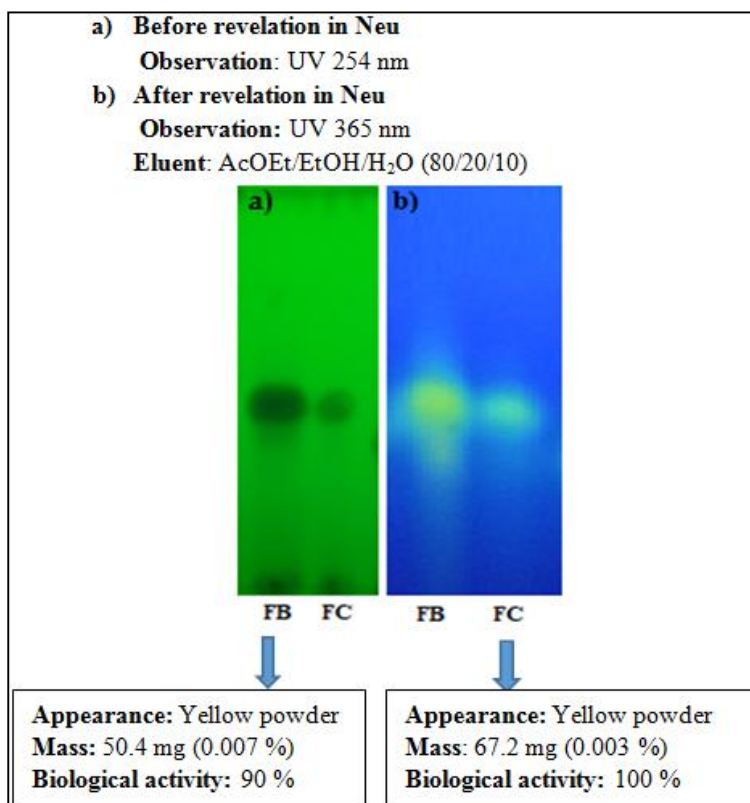
The bioassay of the ethanoid extract of the leaves of the plant and its nine fractions showed vasodilator activity. This activity was more interesting with F1, F6, F9 fractions; quite significant with the F3, F4, F5 and F8 fractions

and very weak with the F2 and F7 fractions (Fig. 3). However, the best activity (100%) was observed with the fraction F9 (brown wax).

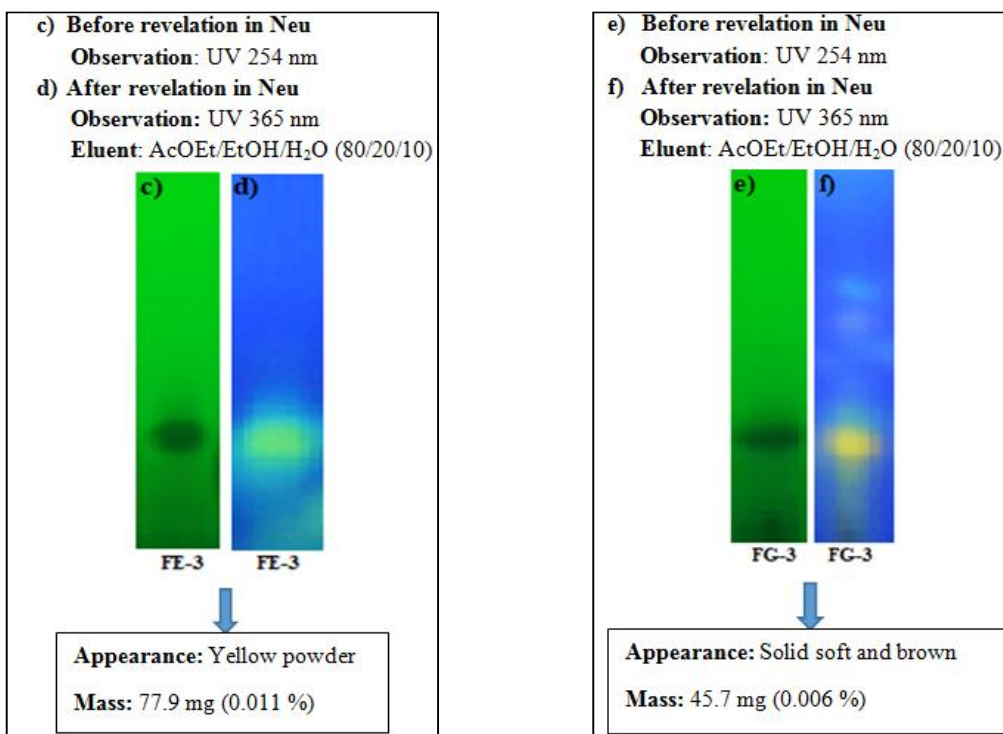
The subfractions F9-1, FB, FC, FE-1 and FG-3 of the F9 fraction exhibited an interesting vasodilator activity (Figs. 5-7).

Fig. 10, characteristic of the vascular reactivity tests carried out during this study, shows that norepinephrine (1 μM) causes aortic contracture up to a value greater than 2 g. Addition of *Trema orientalis* Blume extract (1 mg/mL) at the time of the contracture plateau causes progressive and slow relaxation of the aorta. The ethanolic extract causes relaxation (97%) of the rat aorta. The 100% relaxation percentages are observed with the fractions F1, F6 and F9 obtained from the ethanolic extract (Fig. 3).

Likewise, this relaxation percentage is also observed with the fractions F9-1 and F9-5 coming from the fraction F9 (Fig. 5).



Chromatogram No. 3: TLC polyphenols of FB and FC subfractions



Chromatogram No. 4. TLC polyphenols of FE-3 subfraction

Chromatogram No. 5. TLC polyphenols of FG-3 subfraction

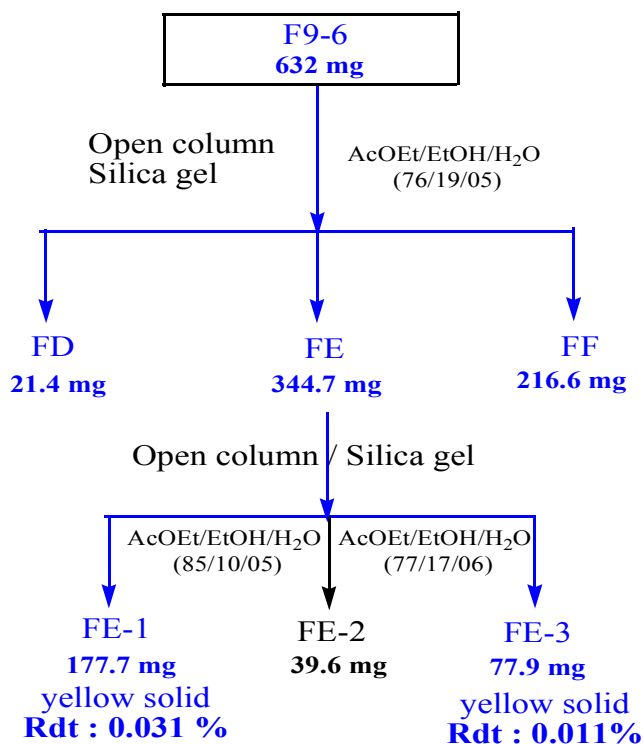


Fig. 8. Fractionation scheme of fractions F9-6

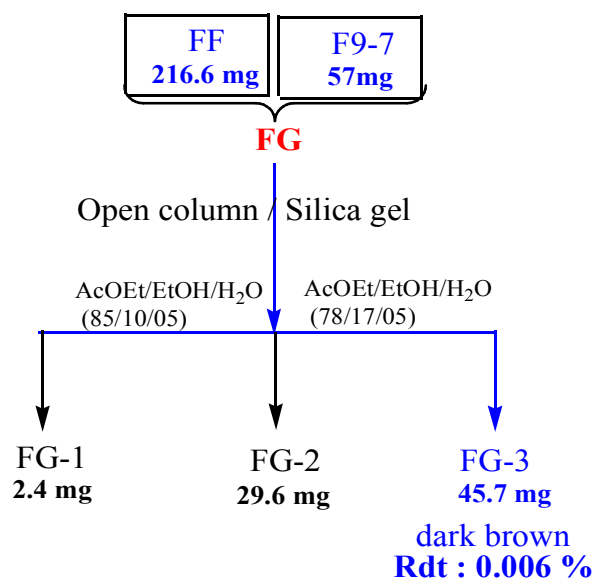


Fig. 9. Fractionation scheme of fraction FG

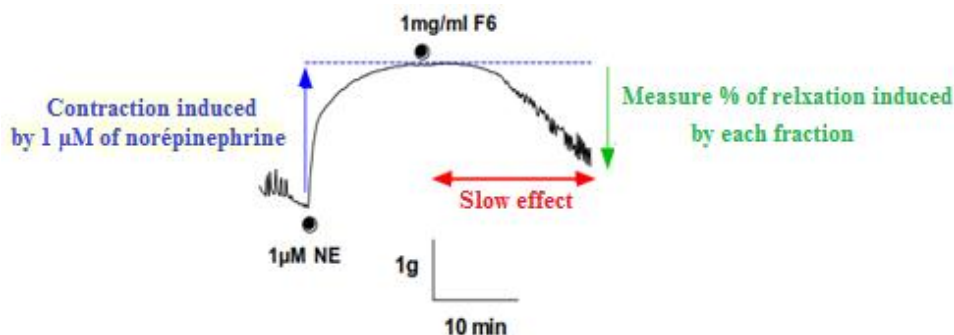


Fig. 10. Example of the contracture of the aorta up to a value greater than 2 g norepinephrine (1 μM)

Fig. 7 shows that subfractions, having a single spot on TLC, from F9 and added to the isolated organ vessel. These subfractions provoke a relaxation of the pre-contracted aorta with norepinephrine (1 μM); the effect of fraction FE-3 being faster with a relaxation percentage of 100%.

The results of the present study show that the ethanolic extract and its fractions derived from *Trema orientalis* Blume cause relaxation of the contracted aorta by norepinephrine. These extracts and fractions therefore have vasodilatory effects on the isolated rat aorta. Similar results with other plant extracts have been obtained by other authors Titrikou et al. (1998, 2007), Dongmo et al. (2002), Etou-Ossibi et al. (2016, 2017) and Kadissoli et al. (2016) on the

contracted aorta with norepinephrine [18,19,22, 36,37,38].

The vasodilator effects of the ethanolic extract and its fractions from this plant could explain the use of this plant in traditional medicine to fight against high blood pressure.

The presence of different chemical families (sterols and terpenes, polyphenols) in the leaves of this plant could justify, in this context, its use in traditional medicine [17-21,24,25,34,35,39].

#### 4. CONCLUSION

The ethanolic extract of the leaves of *Trema orientalis* Blume prepared after successive exhaustion of this organ with hexane, chloroform

and ethyl acetate, showed a terpenoids dominance in fractions F2 to F3 and polyphenols in fractions F7 to F9. The fractionation of F9 allowed to obtain the five subfractions (F9-1, FB, FC, FE-3 and FG-3) that could be compounds pure on TLC. Fractionation was accompanied by a biological activity. Fractions with the best biological activities allowed further fractionation to obtain a fraction with a single spot on TLC. Five obtained fractions characteristic of polyphenols, in particular flavonoids on TLC, showed good vasodilator activity on the rat isolated aorta. The fractionation and biological evaluation of this alcoholic extract and its fractions of the plant species have been of obvious interest for the discovery of new molecules endowed with antihypertensive activity which could lead to the design of new drugs. The identification of these biologically active compounds that contain this organ needs to be deepened in our future work in order to promote this plant.

## CONSENT AND ETHICAL APPROVAL

It is not applicable.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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