Effect of *Tephrosia Vogelii* Hook.f. Leave Extracts on the Isolated Heart of Toad: Hypothesis of the Mechanism of Action

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Effect of *Tephrosia Vogelii* Hook.f. Leave Extracts on the Isolated Heart of Toad: Hypothesis of the Mechanism of Action

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**Abstract**

The ichthyotoxic plants contain substances, which could present a danger to the consumers of caught fish with it. The isolated heart of toad was used in this experimentation according to the modified method of Langendorff. It was a question of having collected the filtrate obtained from the grinding of leaves dried by *Tephrosia Vogelii* in Lock-Ringer, in perfuse the isolated heart of toad with solutions of increasing concentrations by this filtrate. Then to measure the various peaks describing the variations of the contractions and the frequencies of the heart by means of a recorder on paper. Reference solutions were also used except the foliar extracts of *T. Vogelii*. *T.vogelii* (10$^{-3}$ to 10$^{-2}$mg/ml) reduce the amplitude of contraction (negative inotropic) and frequency (negative chronotropic) of the isolated heart of toad; of which both are in a dose-dependent manner (increased and cumulative). The threshold of the effect on contraction and frequency were 3x10$^{-3}$mg/ml and 2x10$^{-3}$mg/ml respectively with maximal effect being 10$^{-2}$mg/ml for contraction and frequency. The rotenone (10$^{-6}$M) just like *T.vogelii* (5.5x10$^{-3}$mg/ml) decreases the contraction and frequency whilst ATP (10$^{-6}$M) increases the two parameters. In presence of the rotenone or *T.vogelii*, the positive inotropic effect induced by ATP is reduced and a negative chronotropic effect is observed. We established that there is an antagonism between the action of the rotenone and the ATP; and that would be via the calcium. The effect of *T.vogelii* on the isolated heart of toad would have been cause by the rotenone.

**Keywords:** ATP, calcium, ichthyotoxic plants, rotenone.
Introduction

The use of ichtyotoxic plants is a common practice of the peasant populations to capture fishes (Petitjean et al., 1993, Mounzéo et al., 1997, Ibrahim et al., 2000). Unfortunately plants ichtyotoxic as Tephrosia vogelii or Justicia extensa, keep their toxicity after the cooking of these fishes (Ibrahim et al., 2000). It is thus necessary to expect that the active constituent involved in the toxicity, infers unwanted effects at the consumers "of captured fishes" from this traditional method. And we think that this active constituent involved in the toxicity could be rotenone. These plants being very rich in rotenone. Indeed, Petitjean et al., (1993) demonstrated that Tephrosia linearis, plant in rotenone toxin for the fish, represented a danger for the man and for the warm-blooded animals. This specie has abortive properties on gestates cows. Mounzéo et al., (1997) pointed out that the consumption of fishesished from Tephrosia vogelii, fishing poison, was not recommended to the women encircled to avoid that the children who will be born develop various diseases such as the epilepsy and the diarrhea. T. vogelii is known "as plant in rotenone" and the toxic effects from extracts of these leaves were already shown on various aquatics bodies (Haag, 1931, Feinstein et al., 1953, Agbon et al., 2004, Ekanem et al., 2004). Inquiries led by Buckingham showed that the rotenone was toxic for the warm-blooded animals (dogs, cats, pigs, sheeps, cows and chickens) (Buckingham, 1930, WHO, 2007). Haag (1931) underlined that this substance caused the death of guinea pigs.

Knowing that T. vogelii is a "plant in rotenone" (Haag, 1931, Feinstein et al., 1953, Agbon et al., 2004, Ekanem et al., 2004), we thought of using the rotenone of synthetize (10-6 M) as reference substance to compare its effects to those of the foliar extracts of T. vogelii. We know that the state of the calcic channels, the activities of the exchanges Na⁺-Ca²⁺, activities of the ions Ca²⁺, of the ATP and the oxygen are very important factors in the process of cardiac contraction (Huxley, 1954, Endo, 2004, Endo, 2006). Indeed, Daut et al., (1994) showed that the heart of mammal consumes more oxygen by gram of fabric than quite different organ of the body. The rotenone leads the reduction of the consumption of oxygen and the synthesis of the adenosine triphosphate cellular (ATP) of the transport chain of electrons mitochondrial during the oxidative phosphorylation (Dickman et al., 1990, Shabalina et al., 1995, Bates et al., 1996, Robertson et al., 2008). The rotenone also allows to reduce the concentration of ions Ca²⁺ cytoplasmic through the calcic channels (Smith et al., 1990, Mc Hugh et al., 1996).

So, we would like to understand the consequences of the ingestion of toxic matter in the body (in particular in the heart) consumers of fishes captured by means of extract of ichtyotoxic plants. That is why we study: "the effect of Tephrosia vogelii and the rotenone on the isolated heart of toad".

Materials and Methods

The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as indicated in the US guidelines (EEC Directive of 1986; 86/609/EEC).

Animal Preparation

The isolated heart of toad was used in this experimentation according to the method modified by Langendorff (1895). From the dissection of a toad, the heart is extracted from the body of the animal. Then the point of the heart is hung on a crowbar connected with a device associated with a paper recorder to collect mechanical reactions of the heart. 12 hearts of toad were used during this study.

Vegetal Preparation

Tephrosia vogelii Hook.f. (Fabaceae) was used to measure. This plant was identified and authentified by Dr Mounzéo Botanist. A specimen was collected in a vegetables garden of Haut-ogoué province in Gabon and deposited with the herbarium of the Department of Biology of the USTM. Having collected the branches of Tephrosia vogelii, behind the compartments of house or in forest, these are immediately aired in the hanging sun three in four days to insure their preservation.
During the study time, leaves collected by the branches of *T. vogelii*, dried, are crushed in the mortar. Then the obtained homogenate is dissolved in a solution of Lock Ringer which is then filtered.

The various concentrations of *T. vogelii* (10⁻³ to 10⁻² mg/ml) used resulted from a solution of 25 mg of the foliar extract of *T. vogelii* in 100 ml of Ringer. The solution obtained was used as experimental medium and Lock-Ringer as control.

**Pharmacological Substances**

A solution of ATP (10⁻⁶M) in Lock-Ringer (solvent) and a solution of rotenone (10⁻⁶M) (Sigma-Aldrich, St. Louis, MO, USA) in DMSO 4% (solvent) were used.

**Experimental Procedure**

The variations of contraction (inotropic effect) and frequency (chronotropic effect) (relative medium Ringer as reference) were measured by a recorder paper (Society Jeulun, France) in a simultaneously acting manner of concentration and time to administer substance for all protocols to increase concentration and cumulative for each isolated heart. For the results of the normalized average of 12 isolated hearts of toad. Results are presented in the form of curve (the curve action – dose is obtained after a period of stabilization of 60 minutes) and histograms. The obtained values were expressed by means ± SD. Statistical significance was tested using student’s test and probabilities of 5% (0.05) were considered significant (software used is Excel 2003).

**Results**

**Dose-Response Effects of Dry Leaves of Tephrosia Vogelii**

After 60 minutes equilibration period in Lock-Ringer’s solution, the isolated heart was perfused with concentrations of dry leaf extracts varying from 10⁻³ to 10⁻² mg/ml in order to determine the limiting dose of the effect of *T. vogelii* on this preparation.

In the study of dose-response, the effect of the action of *T. vogelii*, which begins after 5-8 minutes, is maximal at 20 minutes for each concentration. The results obtained are showed in figure 1. No effect was observed between 10⁻³ – 2.10⁻³ mg/ml on the amplitude of contraction.
However, at $3.10^3\text{mg/ml}$, amplitude of contraction decreased by about 1.4%, while the frequency begins to decrease of 5% at $2.10^3\text{mg/ml}$. The maximal decrease was at $10^2\text{mg/ml}$ for the 50% contraction and the 75% frequency, because after this dose, there is a toxic effect (arrhythmic). Figure 1 shows that the effect of *T. vogelii* was more important on the frequency. Our experiments consist in studying the mechanism of action of the active constituents contained in *T. vogelii* with effects on the contraction and frequency of the isolated heart of toad. Hence, we have chosen $5.5x10^{-3}\text{mg/ml}$ (ID$_{50}$ of frequency).

**Effects of Tephrosia Vogelii (5. 5x10$^{-3}$) mg/ml and ATP (10$^{-6}$M)**

Figure 2A shows that $5.5.10^{-3}\text{mg/ml}$ of *T. vogelii* decrease the contraction by 23.34% (figure 2A-c1) and the frequency by 14.25% (figure 2A-f1) of the heart. This effect begins at 5 min and is maximal at 15 min. When the heart was perfused with a solution of ATP ($10^{-6}$M) without *T. vogelii*, an increase in the contraction by 51.17% (figure 2A-c2) and the frequency by 12.11% (figure 2A-f2) was observed. Time onset effect is 5 min and maximal time between 15 - 20 min.

**Effects of Rotenone (10$^{-6}$M) and ATP (10$^{-6}$M)**

The results (figure 2B) show that, when the heart was perfused with a solution of rotenone ($10^{-6}$M), the contraction and frequency decrease by 33.74% (figure 2B-c1) and 21.98% (figure 2A-f1) respectively. This effect was onset at 7 min and maximal at 10 min. When this preparation was perfused with a solution of ATP ($10^{-6}$M) without rotenone, the contraction and frequency increased by 38.93% (figure 2B-c2) and 7.61% (figure 2B-f2) respectively relating to medium Ringer. Time onset effect is 5 - 6 min and maximal time is at 10 min. DMSO 4% (as solvent of rotenone) had no effect on the toad heart isolated after 60 min (results not presented).
**Effects of Tephrosia Vogelii (5.5x10^{-3}) mg/ml and Tephrosia Vogelii (5.5x10^{-3}) mg/ml + ATP (10^{-6}M)**

Figure 3A illustrates that contraction and frequency decrease by 15.66% (figure 3A-c1) and 13.27% (figure 3A-f1) respectively when the heart was perfused with a solution of *Tephrosia vogelii* (5.5x10^{-3}) mg/ml (time onset effect is between 5 - 6 min and maximal time between 15 - 20 min). When perfused with a solution of *T. vogelii* (5.5x10^{-3} mg/ml) and ATP (10^{-6}M), an increase of contraction by 35.90% (figure 3A-c2) and a decrease of frequency by 26.19% (figure 3A-f2) was observed, relating to medium Ringer. Whereby, this effect begins after 5 min and maximal between 15 – 20 min.

**Effects of Rotenone (10^{-6}M) and Rotenone + ATP (10^{-6}M)**

The figure 3B shows that rotenone (10^{-6}M) decreases contraction and frequency by 22.74% (figure 3B-c1) and 14.04% (figure 3A-f1) respectively. The time onset effect is 5 min and maximal time between 10 - 15 min. When perfused with a solution of both rotenone (10^{-6}M) and ATP (10^{-6}M), an increase of contraction by 15.14% (figure 3B-c2) and a decrease of frequency by 16.71% (figure 3B-f2) was observed, relating to the medium of reference (Lock - Ringer), whereby this effect begins at 5 min and maximal between 10 – 15 min.
Effects of ATP (10^{-6} M) and Tephrosia Vogelii (5.5\times 10^{-3} mg/ml)

When the heart was perfused with a solution of ATP (10^{-6}M), there is increase of both contraction and frequency by 46.58% (figure 4A–c1) and 5.87% (figure 4A–f1) respectively. The time onset effect is 5 – 7 min and maximal between 10 – 15 min. When perfused by a solution of T. vogelii (5.5\times 10^{-3} mg/ml) without ATP, the inotropic positive induced by ATP (10^{-6}M) was reduced from to 31.08% (figure 4A–c2) whereas the frequency decreased by 23.65% (figure 4A–f2:105.87% to 82.22%). Time onset effect is 8 min and maximal time between 15 – 20 min.
**Effects of ATP ($10^{-6}$M) and Rotenone ($10^{-6}$M)**

The results of figure 4B show that when the preparations are perfused by a solution of ATP ($10^{-6}$M), the amplitude of both contraction and frequency increased by 35.52% (figure 4B–c1) and 5.16% (figure 4B–f1) respectively. The effect begins at 7 min and maximal at 15 min.

When these preparations were perfused by a solution of rotenone ($10^{-6}$M) without ATP, there is decrease of both contraction by 33.52% (35.52% to 2%; Figure 4B–c2) and the frequency by 26.22% (figure 4B–f2:105.16% to 78.94%). This is the chronotropic negative related to chronotropic positive effect induced by ATP ($10^{-6}$M). Onset time effect is 5 min and maximal time at 15 min.

**Discussion**

Our results show that leave extracts of *T. vogelii* ($10^{-3}$-$10^{-2}$mg/ml) decreases the amplitude of contraction (negative inotropic effect) and the frequency (negative chronotropic effect) isolated heart of toad and this is in a dose-dependent manner (increasing and cumulative; figure 1). We note that no effect of *T. vogelii* was observed for $10^{-3}$ and $2.10^{-3}$ mg/ml on the contraction, at $3.10^{-3}$ mg/ml it decreases (1.4%; threshold of the effect; negligible but revealing) whereas its effect on the frequency (which decreases: 5%), starts at $2.10^{-3}$ mg/ml. The maximum reduction was considered at $10^{-2}$ mg/ml for the contraction and frequency, because beyond this dose cardiac arrhythmias appears, which would harden the rigorous evaluation of the parameters considered for analysis of the mechanism involved in the effect of *T. vogelii*. For each concentration of leave extracts of *T. vogelii*, the effect begins between 5 - 8 min and is maximum at 20 min. Thus, we observed that at $10^{-2}$mg/ml the contraction decreased by 50% and the frequency by 75% (figure 1).

*T. vogelii* by its action has an effect in a dose-dependent manner (increasing and cumulative) decreasing the activity of isolated heart of the toad.
(particularly of the negative inotropic and chronotropic effects). Many authors have already worked on fishing poison (Walker, 1951, Petitjean et al., 1990, 1992, 1993, Ibrahim et al., 2000, Agbon et al., 2004, Ekanem et al., 2004, Neuwinger, 2004, Robertson et al., 2008, Akpa et al., 2010). The results on figure 1 illustrate that the frequency seems to be more affected than the contraction when the heart was perfused in a dose-dependent manner. For this reason, we have chosen 5.5x10^3 mg/ml as concentration of T. vogelii (which reduced 50% the frequency or ID₅₀, whereas with this concentration the contraction decreased only by 22.40%). The continuation of our investigations will consist to study the mechanism of the active constituent(s) of the leave extracts of T. vogelii that cause the above effects. The synthesized rotenone (10^6 M)(Harvey et al., 1999) was taken as a reference substance in order to compare its effects with those of the leave extracts of T. vogelii. As, T. vogelii is known as 'rotenone plant' (Haag, 1931, Feinstein et al., 1953, Agbon et al., 2004, Ekanem et al., 2004.). Because of the high percentage of rotenone (25%) than other substances (rotenoids) in its composition (Jones et al., 1933).

Our results in figure 2B show that at 10^6 M, the synthesized rotenone reduce the contraction of both (figure 2B-c1: 33.74%) and frequency (figure 2B-f1: 21.98%). These effects of the rotenone are similar to those obtained in presence the leave extracts of T. vogelii (5.5x10^3 mg/ml). Indeed, we note that T. vogelii reduces contraction (figure 2A-c1: 23.34%) and frequency (figure 2A-f1: 14.25%) of the isolated heart of toad. The effect of T. vogelii would imply that the rotenone contained in its composition without excluding the presence of the rotenoids (Feinstein et al., 1953, Nwude, 1982), which could act in synergy or not with the rotenone.

Some studies (Wijburg et al., 1990, Mc Hugh et al., 1996, Hasegawa et al., 1997, Li et al., 2003, Rizzardini et al., 2006) pointed out that the rotenone, binding at a specific site (complex I) of the respiratory chain of the mitochondria causes the reduction of Adenosine Triphosphate (ATP) synthesis. This reduction of the synthesis of ATP would also cause the diminution in the cytosolic concentration of calcium (Smith et al., 1990, Mc Hugh et al., 1996) as well as the oxygen uptake (Dickman et al., 1990, Shabalina et al., 1995, Bates et al., 1996, Fariss et al., 2005).

However we know that the state of the calcium channels, of the activity of the exchange Na⁺-Ca²⁺, the Ca²⁺ ions, the ATP and oxygen are very important factors in the processes of contraction (Huxley, 1954, Blink et al., 1986) and frequency (Endo, 2004, Endo, 2006). For contraction of heart muscles, a Ca²⁺ influx from extracellular is required (Sperelakis et al., 1993, Endo, 2004, Toshes et al., 2004, Endo, 2006,) and that the cardiac contraction force depends on the rate of the heart (Endo et al., 1970). Our results (figures 2A – 2B) are in agreement as the work of Endo (2004) which showed that inhibition of calcium channels reduce the heart rate (negative chronotropic effect; figure 2A-f1: 14.25% and figure 2B-f1: 21.98%). This consequently, involves the reduction of cytoplasmic [Ca²⁺] inducing the negative inotropic effect (figure 2A-c1: 23.34%; figure 2B-c1: 33.74%).

Thus, the rotenone binding at the complex I (Finel et al., 1992, Hasegawa et al., 1997) of the respiratory chain of the mitochondria would induce the reduction of the ATP synthesis (Chambers et al., 1990, Weinbertg et al., 1990, Finel et al., 1992). That decrease of the cytoplasmic ATP would involve not only inhibition of calcium channels (Sperelakis et al., 1993, Mc Hugh et al., 1996) leading to the negative chronotropic effect (figure 2B-f1), but it would also be lower the activity of the exchange Na⁺-Ca²⁺ (Smith et al., 1990, Haworth et al., 1992). Inhibition of the calcium channels and the Na⁺-Ca²⁺ exchange would thus cause the decrease in the cytoplasmic ionic Ca²⁺, inducing the negative inotropic effect (figure 2B-c1) of T. vogelii on isolated heart of toad.

The diminution of the synthesis of ATP seems to be the cause of amplitude of contraction and frequency decline. In this experimental method, it is not possible to directly quantify the variation of the rate of ATP in the cytoplasm under the effect of the leave extracts of T. vogelii or rotenone. However, the impact of the modifications of Ca²⁺, ATP and oxygen concentration could be evaluated indirectly considering the consequences induced by the
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extracts of T. vogelii or rotenone on the variation of the contraction amplitude and frequency of the isolated heart of toad.

In this study, we focused only on the variation of the diffusion of calcium through the calcium channels and the cytoplasmic concentration of ionic calcium. In order to compensate the rate of calcium reduced by inhibition of calcium channels, which is cause by the reduction in cytoplasmic [ATP] following the action of the rotenone or of T. Vogelii. We used the synthesized ATP (extracellular) to stimulate the calcium channels ‘partly’ involved in the process of the frequency (Scamps et al., 1990) and to induce the increase in the cytoplasmic \([\text{Ca}^{2+}]\) (mechanism of the contraction). Results of figure 2 shows that, the ATP permits a positive inotropic (figure 2A-c2: 151.19% and figure 2B-c2: 138.93%) and positive chronotropic effects (figure 2A-f2: 112.11% and figure 2B-f2: 107.61%) on the isolated heart of toad. Scamps et al., (1990) studied the heart of rat and showed that the extracellular ATP binds to its membrane receptor (P2), increases the concentration of cytoplasmic ionic \([\text{Ca}^{2+}]\) hence stimulating the calcium channels. Endo noted that the stimulation of the calcium channels (positive chronotropic effect) makes an increase in cytoplasmic \([\text{Ca}^{2+}]\) inducing positive inotropic effect (Endo, 2004).

These works (Endo, 2004, Scamps et al., 1990) are similar to those of our results (figure 2). However (see figure 3), we observed that the positive inotropic effect due to the action of the ATP is reduced from 151.19% to 135.90% in the presence of T. vogelii (3A-c2 figures) and from 138.93% to 115.14% in the presence of the rotenone (figures 3B-c2), whereas we noted a negative chronotropic effect (figure 3A-f2: from 112.11% to 85.92% in the presence of T. vogelii and figure 3B-f2: from 107.61% to 85.96% in the presence of the rotenone).

The rotenone or T. vogelii cause the reduction of ATP synthesis leading to inhibition of calcium channels (Sperelakis et al., 1993, Mc Hugh et al., 1996) which finally induces the negative chronotropic effect and reduction of the positive inotropic effect. In the results of figure 4, the positive inotropic effect cause by the ATP is reduced from 146.58% to 131.03% by T. vogelii (figure: 4A-c2) and almost completely by the rotenone (figure 4B-c2: from 135.52% to 102.00%), while the positive chronotropic effect induced by the ATP becomes negative (decrease from 106% to 72.35%) in the presence of T. vogelii (4A-f2 figure) or from 105% to 78.78% in the presence of the rotenone (figure 4B-f2). This compare well to the effect induced by the ATP, confirming the inhibitor effect of T. vogelii or the rotenone (reduction of the ATP synthesis), and that the frequency would be most affected as our results of figure 1 shows. At 5.5x10\(^{-3}\)mg/ml of T. vogelii, the frequency is reduced to 50% while, the contraction decreases only by 22.40%.

The effect of T. vogelii (5.5x10\(^{-3}\)mg/ml) was reversible after approximately 30 min, while for the ATP (10\(^{-6}\)M) and for the rotenone (10\(^{-6}\)M) were 30 - 45 min and 60 min respectively, when the preparations were perfused again with normal.

Conclusion

From these studies, it can be concluded that, the rotenone binding at complex I, cause inhibition of the activity of the complex which involves partial reduction of the cytoplasmic concentration of the ATP. This leads to inhibition of calcium channels and the decrease of the concentration of ionic calcium inducing respectively the negative chronotropic effect and reduction of the positive inotropic effect.

Like the synthesized rotenone, the leave extracts of T. vogelii could cause the reduction of frequency and decrease in amplitude of the contraction of the heart of the consumer.

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