# EVIDENCE OF PHOSPHOLIPASE C SIGNALING PATHWAY ACTIVATION BY *BIDENS PILOSA* LEAF AQUEOUS EXTRACT ON THE RAT PRIMED-ESTROGENISED MYOMETRIUM

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**Summary**

In this study, we tested the effect of *Bidens pilosa L.* leaf aqueous extract on the $G_{q/11}$/$PLC_\beta$ signaling pathway of rat primed-estrogenized uterine strips. We found that *Bidens pilosa* alone induced a concentration dependant increase in the contractile activity in normal Krebs's solution. Pre-incubation with some $Gq_{/11}$/PLC signaling pathway agonist and antagonists showed significant ($P \leq .05$) and maximal inhibition potencies observed in presence of a specific α1-adrenergic receptor antagonist 10µM Phentolamine: -537.66%, -34.42% and -185.70% on the amplitude , tonus and rate of oscillations respectively. Maximal potentialization potencies were observed in presence of Atropine, a muscarinic receptor antagonist on the tonus (+231.26%) and on the rate of contractions by Phenylephrine, a α1-adrenergic receptor agonist (+155.87%). Tetrodotoxin, a $Na^+$ channel blocker, also strongly inhibited the rate of oscillations up to -111.75%. The phospholipase C inhibitor (U-73122 10µM) and the Inositol 1,4,5 triphosphate (IP3) receptor inhibitor (Heparin 900UI/ml) inhibit *Bidens pilosa* induced myometrium contractile activity at potencies (amplitude -35.48% and rate of oscillation -32.43% respectively) inferior to that of the α1-adrenergic receptor inhibitor. These results indicate that α1-adrenergic receptors, and consequently $G_{q/11}$/$PLC_\beta$ signaling pathway represent the major activated signaling pathway of the induced contractile activity by *Bidens pilosa* leaf aqueous extract on the rat primed-estrogenized myometrium.

**Key Words: *Bidens pilosa L.*, $G\alpha_{q/11}$/$PLC_\beta$ modulators, Rat, Myometrium**

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## Introduction

The tropical Asteraceae weed *Bidens pilosa Linné* (*B. pilosa*), is used world-wide to treat various diseases because of its antimicrobial, antiglyceamic, anti-inflammatory effects amoung many others. In Cameroon, the aqueous extract of *B. pilosa* is used by traditional birth attendants as a labour stimulator in order to facilitate delivery. In rat and human myometrium at the end of pregnancy before labour initiation, under estrogens control, there's an increase in density and affinity for ligands of various receptors that regulate the uterine sensitivity and involved in the initiation of labour (1). Among many signaling pathways, the phospholipase C pathway is one of the most requested during receptor activation by agonists and, it's also the most represented in various receptors families that coexist on the uterine membrane (2).

According to many workers (1-3), at the end of the pregnancy, several receptors and channels are profusely expressed at the caudal uterine horn and cervix due to estrogens and other factors. Among them, receptors coupled to $G_{\alpha q/11}$ protein which activates the phospholipase C transmembrane enzyme such as α1-adrenergic, Oxytocin (OTR), muscarinic and channels [VOCLs, $Na^{+}$] enhance the uterine contractility during labour. On contrary to α1-adrenergic, ocytocin (OTR) receptors, the density of muscarinic receptors is not affected but their sensitivity to Acetylcholine enhance in presence of hormones imbibition (estrogens and factors) (1, 4).

Earlier studies (5) demonstrated that the phospholipase C (PLC) system plays a pivotal role on the regulation of uterine contractility. The transmembrane enzyme phospholipase $C_\beta$ ($PLC_\beta$), one of the 3subtypes meet in the uterine muscle, is the most expressed at the end of the pregnancy. $PLC_\beta$ is the first enzyme that initiates the cascade of events involved in the increase of cytosolic $Ca^{2+}$ with finally a MLCK (myosin light chain kinase) dephosphorylation leading to contraction. Thus, drugs that link to the amino acid $SER^{1105}$ of the α unit of protein $G_{\alpha q/11}$ binding site affects it function (6). The $PLC_\beta$, after been activated by the $\alpha_q$ or $\alpha_{11}$ subunits; hydrolyzes the myometrial membrane substrat phosphatidylinositol 4, 5-bisphosphate ($PIP_2$) to produce two intracellular second messengers: diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate ($InsP_3$). $IP_3$ releases calcium ($Ca^{2+}$) from internal stores. The Inositol [1, 4, 5] triphosphate ($InsP_3$) is the last product issued from the cascade of events after agonist binding on a $G_q$-protein/ PLC receptor. The $InsP_3$ receptor is a receptor-channel located on the sarcoplasmic reticulum membrane and its release $Ca^{2+}$ (puffs) to potentiate the contractile force via membrane depolarization and increased the voltage gated $Ca^{2+}$ channels (VOCLs) activity (7-9).

The subsequent increase of intracellular free $Ca^{2+}$ via a calciprotein transporter (Calmodulin) activates the myosin light chain kinase (MLCK) which is the key enzyme that promotes the interaction between contractile proteins: actin and myosin, resulting in contraction.

Different extracts of *B. pilosa* leaves extract have been tested earlier on normotensive/*hypertensive* rats by (10-12) and they demonstrated that it decreased systolic blood pressure up to 38.96% and induced hypotension up to 34.13% accompanied by a 23.68% decrease in heart rate. The authors also thought that the relaxant effect observed on rat aortic strips Norepinephrine and KCL-induced takes place by a blocking effect on Voltage-gated L-type calcium channels with is due mainly through adrenergic receptors (105%).

In this report, we tested *B. pilosa* leaves aqueous extract on the Phospholipase C signaling pathway by antagonizing its effect in presence of some modulators from membrane receptors to the reticulum sarcoplasmic $InsP_3$ receptor.

## Material and Methods

### Preparation of the Aqueous Extract:

*Bidens pilosa* plants were collected from Yaounde's suburb (Nkolbisson) from July-November 2005. A voucher specimen N° 65112/H.N.C was authenticated and deposited in the National Herbarium of Cameroon. Following the traditional birth attendants (TBA) use, two hundred grams of sun-dried leaves were boiled in one litre of water for twenty minutes, filtered and freeze-dried giving 38g of a brownish powder. A stock solution of 25mg/ml in bi-distilled water was prepared daily.

### Tissue Preparation:

Adult virgin female Wistar rats, weighting 200-250g, were purchased from Janvier Breeding Centre (France). Twenty-four (24) hours before the experiment, animals received an intraperitoneal injection of $17^{\beta}$ Oestradiol Benzoate [20µg × Kg-1] then euthanized by 1min inhalation of carbon dioxide ($CO_2$). Rats were treated following the international ethical conduct of animals's treatment (13).

### Isometric Contraction Measurements

Uterine horns were removed from the dam, getting rid of adhering fats and 1mm strips were mounted between two stainless steel clips in a vertical 5ml organ baths filled with Krebs Heinseleit (KH) solution: [(mM): NaCl 118.4; KCl 4.7; $KH_2PO_4$, 1.2; $MgSO_4 7H_2O$, 1.2; $C_6H_{12}O_6$, 11.1; $NaHCO_3$ 25.0 and $CaCl_2$ $2H_2O$, 2.5; p.H 7.4 (NaOH)], warmed at 37°c, bubbled with carbogen (5%$C0_2$, 95%$O_2$) and equilibrated as previously described by (14, 15) Preparations were challenged by administration of a maximal effective concentration of Acetylcholine (Ach), 100µM final concentration in the bath. The Ach-induced contraction was considered as a reference standard contraction and used to normalize the following contractile responses. Uterine strips were rinsed 3 times and kept not stimulated until stable and regular spontaneous contractions appeared for a further 30min before tests.

For each set of experiment, a ring obtained from the same horn was used as a paired temporal control. Different volumes of the aqueous stock solution were cumulatively injected in the bath at ten-minute intervals for final concentrations of 0.03; 0.09; 0.22; 0.47; 0.97; 1.97; 3.97mg/ml after incubation with a volume of a stock solution of each pharmacological substance, giving final concentrations of: Tetrodotoxin 1µM (30min), Atropine 10µM (30min), Phenylephrine 10µM (40min), Phentolamine (40min), U-73122 10µM (30min), and Heparin 900UI/ML (60min).

### Chemicals and Drugs

17β Oestradiol benzoate, Phentolamine hydrochloride (Phentol), Phenylephrine hydrochloride(16), Acetylcholine chloride (Ach), Atropine sulphate, 1-[6-[((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl) amino]hexyl]-1H-pyrrole-2,5-dione (U-73122), Heparin sodium salt and chemicals for the Krebs's solution were purchased at Sigma Aldrich Chemicals (Saint Quentin Fallavier, France). Ethanol, Dimethyl Sulfoxide (DMSO) was used for drug's dilution whenever appropriate.

**Statistical Analysis**

The statistical significance of difference between control and treated strips were analyzed following the One-Way Analysis of Variance (ANOVA) using all pairwise Dunnett's or Tukey's methods whenever appropriate. The difference between two means between treated and control strips were assessed by the *t*–test. Data are reported as the mean ± S.E.M. for *n* number of experiences. Each *n* represent $n^3$. The significances were determined at P≤.05. All statistics were performed using Sigma Stat version 2.0; Curves drawing and $EC_{50}$ by Sigma Plot version 9.0.

**Parameters Calculation**

*Amplitude of Oscillations:* $= [(A_c-A_0)/ (A_{Ach}-T)/100]$
*Tension:* $= [(T_c-T_0)/ (A_{Ach}-T_0)/100]$
*Frequency of oscillations:* $= [(F_c-F_0)\times100]$

$A_c$ = Amplitude recorded during *B. pilosa* cumulative addition in the bath in presence or absence of the pharmacological drug at *c* concentration.

$A_0$ = Amplitude recorded after the equilibrating period which follows wash of the reference Ach contraction (control strip) and after the effect of the pharmacological substance (treated strip).

$A_{Ach}$ = Amplitude of the reference contraction of Ach 100µM

$T$ = Basal tone recorded before recording the reference Ach contraction.

$T_0$ = Basal tone recorded after the equilibrating period who follows wash of the reference Ach 100µM (control strip) and after the effect of the pharmacological substance (treated strip).

$F_c$= Frequency recorded during *B. pilosa* cumulative addition in the bath in presence or in absence of the pharmacological drug at *c* concentration.

$F_0$ = Frequency recorded after the equilibrating period who follows wash of the reference Ach 100µM (control strip) and after the effect of the pharmacological substance (treated strip).

Results are expressed in the text as difference between the drug effect and *B. pilosa* effect (%).

## Results

**1/ Effects on $Na^+$ Channel**

In order to see if the induce contractile activity of the aqueous leaf extract of *B. pilosa* could be due to a parasympathetic nerve stimulation and/or due to the uterine $Na^+$ channels opening, 1µM TTX, a specific Na+ voltage gated channel inhibitor (17), was added in the preparation for 30 min incubation. Addition of cumulative concentrations of *B. pilosa* did not significantly modify (P>.05) the myometrium contractile activity [Figure1]. Whatever, TTX induced a concentration dependent decrease of amplitude of contractions at 0.97mg/ml equivalent to -3.08±3.02% that of the extract alone [$Figure2_{IA}$]. The tonus base line did not change up to 0.22mg/ml but beyond, it increased with a maximal value of +11.78±2.99% 0.97mg/ml. At the highest concentration of 3.97mg/ml, TTX showed a competitive behaviour with a decrease of -3.66±1.48% [$Figure2_{IB}$]. On the rate of contractions, TTX showed a non

significant (P>0.05) competitive antagonism with the highest decrease value of -31.67±0.17% observed at 1.97mg/ml [Figure $1_{IC}$]. The efficacy of the plant extract in presence of TTX was inferior $EC_{50Bp}<EC_{50Bp\text{-}TTX}$ to that of the extract alone on the rate but significantly superior (P≤.05) $EC_{50Bp\text{-}TTX} < EC_{50Bp}$ on the amplitude and the tonus [Table 1].

**2/ Effects on Muscarinic Receptors**

Pre-incubation with Atropine 10µM, a non selective muscarinic inhibitor (1, 18) during 30mn, abolished spontaneous contractions up to the basal tonus [Figure1]. Cumulative concentrations of *B. pilosa* in the bath induced a contractile activity of uterine strips with a significant (P≤.001) concentration-dependant decrease of amplitude equivalent to -20.72±3.05% 3.97mg/ml [Figure$2_{IIA}$]. The tonus increased in a concentration dependant manner from the concentration of 0.47mg/ml with a maximal value representing +24.86±9.14% (P≤.001) [Figure$2_{IIB}$], while a general decrease of the rate of contractions was observed with a peak increase of +9.17±12.56% at 1.97mg/ml (P≤.05), followed by a high inhibition up to -63.33±3.12% at 3.95mg/ml [Figure$2_{IIC}$]. The efficiency of the extract in presence of atropine [Table1] was significantly (P≤.05) higher than that of the extract alone for all parameters.

**3/ Effects on $\alpha_1$-Adrenergic Receptors**

*B. Pilosa* extract tested in presence of the specific α1-adrenergic receptor modulators showed no significant modification in presence of the contractile activity in presence of 10µM PHE, and a contractile activity recovery in presence of 10µM Phentolamine [Figure1], but on regard to the three parameters, uterine strips presented variably behaved. On the amplitude of oscillations, at low concentrations (≤ 0.47mg/ml), PHE showed a weak decrease of -3% while Phentolamine showed an increase with the highest value of +6.46±2.25% observed at 0.22mg/ml. At concentrations >0.47mg/ml, both significantly (P≤.05) inhibited the amplitude with the highest effect observed in presence of Phentolamine: -20.70±6.05% at 1.97mg/ml and -49.97±10.12% at 3.97mg/ml (P≤.05) [Figure$2_{IIIA}$]. For the tonus parameter, up to the concentration of 0.47mg/ml, PHE and Phentolamine presented no tonus variation; from 0.47 to 1.97mg/ml, Phentolamine presented a potentialization (+9.65±5.55%) while PHE exhibited no tonus variation. From 1.97mg/ml, both drugs inhibited the tonus with at values equivalent to -4.98±5.40% and -3.14±3.01% at 3.97mg/ml for Phentolamine and PHE respectively [Figure$2_{IIIB}$]. On the rate of oscillations, Phentolamine presented a general decrease up to -55.71±2.40% at 1.97mg/ml while PHE presented a general increase with a maximal significant (P≤.05) effect of +46.67±14.02% at the same concentration. Both PHE and Phentolamine presented a competitive antagonism to *B. pilosa* [Figure$2_{IIIC}$]. *B. pilosa* efficacy increase non significantly (P>05) in presence of PHE and diminish significantly (P≤.05) in presence of Phentolamine on the amplitude of contractions. On the tonus and rate of contractions, *B. pilosa* efficacy increased but was only significantly different in presence of Phentolamine (P≤.05) [Table 1].

**4/ Effects on Phospholipase C Enzyme (PLC)**

U-73122 have been reported to inhibit PLC enzyme (19, 20). The preparation incubated during 40min with 10µM U-73122, did not completely suppressed the strip's contractile activity. Cumulative concentrations of *B. pilosa* in presence of this selective $PLC_\beta$ inhibitor caused a general decreased of the induced contractile activity of *B. pilosa* effect for

all parameters [Figure1]. The maximal decreases were observed at the concentration of 1.97mg/ml with significant differences of -19.65±0.59% (P≤.001) for the amplitude, -36.29±6.31% for the tonus (P≤.001) and –27.86±8.05% (P≤.05) for the rate of contractions [Figure$2_{IV\text{-}A,\ B,\ C}$]. *B. Pilosa* $EC_{50}$ on the three parameters showed a significant decrease on the amplitude (P≤.05) and on the rate (P≤.001) while it was superior on the tonus (P≤.0001) [Table 1].

**5/ Effects on Inositol [1, 4, 5] Triphosphate Receptor: ($InsP_3$-R)**

Heparin has been found to specifically inhibit the opening of the $InsP_3$-R (7, 21, 22) thus, prevents $Ca^{2+}$ ions movements from the sarcoplasmic reticulum to the cytosol. Addition of 900UI/ml Heparin in the preparation completely abolishes the contractile activity of the uterine muscle without changing the basal tone. Cumulative concentrations of *B. pilosa* extract caused the recovery of the oscillatory activity [Figure1] and variations appeared on regard to each selected parameter. On the amplitude of oscillations, we noted a general non competitive concentration-dependant decrease up to -9.43±3.47% (P≤0.001) at 3.97mg/ml (Figure$2_{VB}$). On the tonus, we noted a weak decrease up to the concentration of 1.97mg/ml and a weak peak increase of +6.97±14.55% (P≤.001) at 3.97mg/ml (Figure $2_{VB}$). The rate of oscillations was also influenced by the presence of Heparin with an increase up to +23.248±3.53% at the concentration of 0.97mg/ml; but from the concentrations of 1.97mg/ml (Figure $2_{VC}$), *B. pilosa* extract showed a significant (P≤0.05) rate decrease of the oscillatory activity of -19.25±6.34% .$EC_{50}$ values of *B. pilosa* effect presented a significant higher activity in presence of Heparin on amplitude (P≤0.001) and rate (P≤0.05) of oscillations while on the tonus the efficacy was significantly (P≤.0001) lower [Table 1].

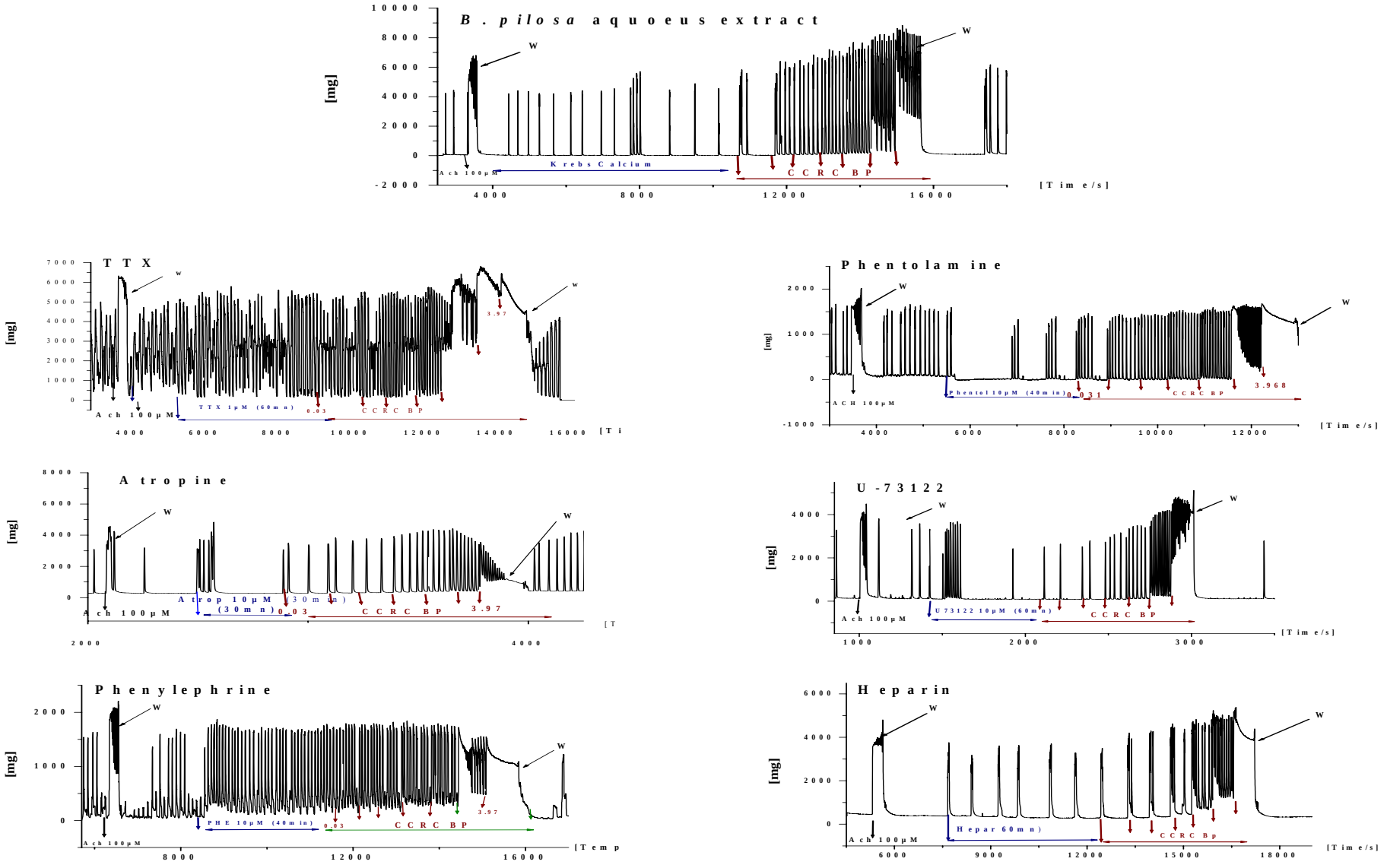


**Figure 1:** Original recordings of the effects of *B. pilosa* leaf aqueous extract alone and in presence of 1µm tetrodotoxin, 10µM Atropine, 10µM Phentolamine, 10µM phenylephryne, 10µM U-73122 and 900UI/ml Heparin on the primed-estrogenized rat uterine strips. Each concentration is representing by an arrow. Ach: acetylcholine, W= wash with Normal Krebs.

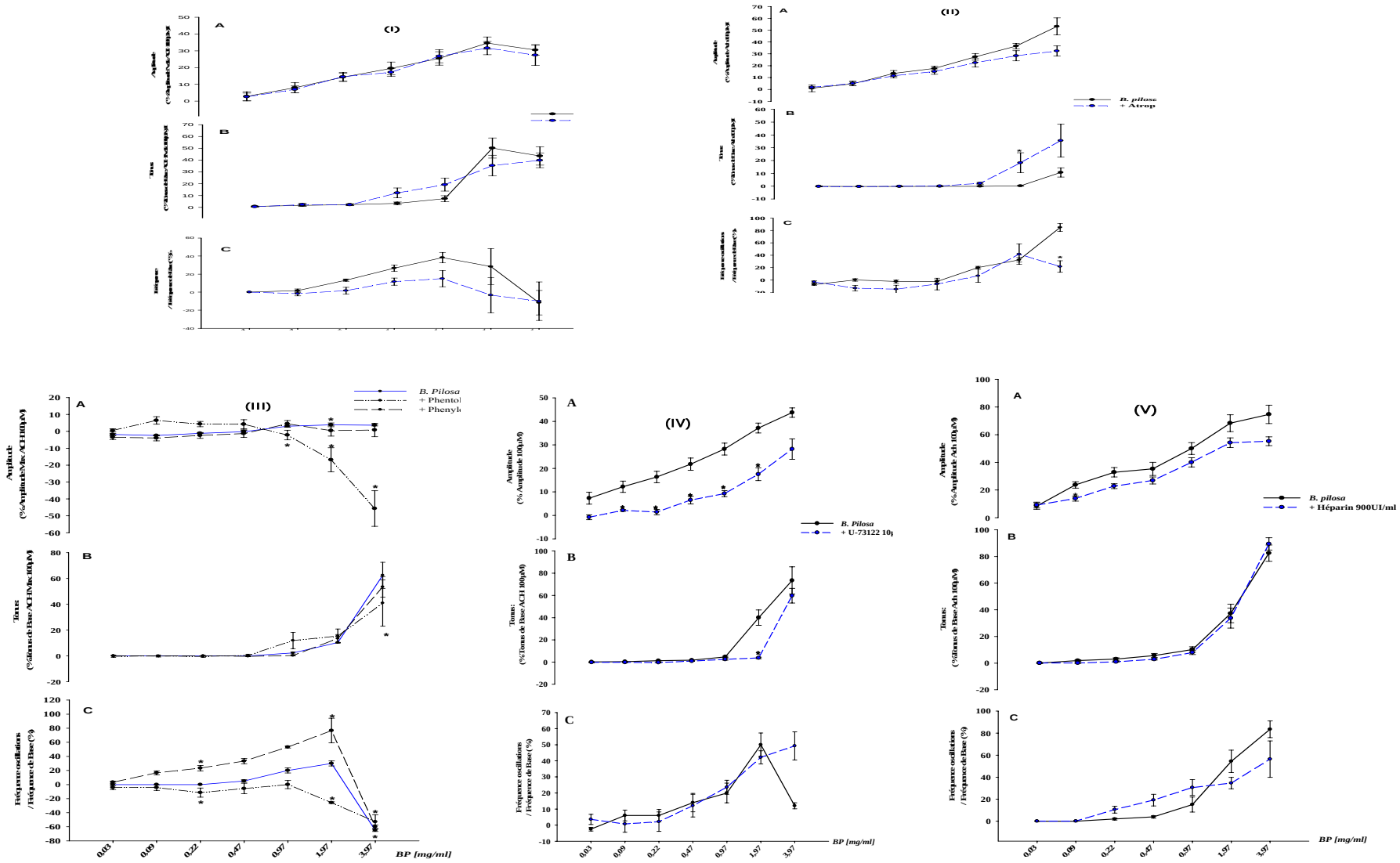


**Figure 2:** Representatives curves of *B. pilosa* leaves aqueous extract effects on Amplitude (**A**), tonus(**B**) and the rate (**C**) on the contractile activity of primed-estrogenized rat myometrium muscle in presence of 1µm Tetrodotoxin (**I**), 10µM Atropine (**II**), 10µM Phentolamine (**III**), 10µM Phenylephrine (**III**), 10µM U-73122 (**IV**) and 900UI/ml Heparin (**V**).

**Table 1:** Different $EC_{50}$ values of leaf aqueous extract of *Bidens pilosa* tested alone and in presence of some phospholipase C signaling pathway modulators on the contractile activity of the rat primed-estrogenized myometrium. T-test: *P≤0.05; ** P≤0.001; ***P≤0.0001.

| *B. pilosa* Aqueous Extract | $EC_{50}$ (mg/ml) | | |
|---|---|---|---|
| | Amplitude | Tonus | Rate |
| + KH | 0.37±0.09* | 1.18±0.71** | 1.19±0.17 |
| + Tetrodotoxin 10µM *(n=7)* | 0.34±0.08* | 0.95±0.11** | 1.79±1.41 |
| + KH | 0.51±0.29* | 2.13±0.26*** | 2.22±0.37* |
| + Atropine 10µM *(n=6)* | 0.43±0.109* | 1.95±0.01** | 0.10±3.32 |
| + KH | 0.93±0.86* | 2.80±0.16*** | 3.64±0.27 |
| + Phenylephrine 10µM *(n=6)* | 0.32±0.48 | 2.18±0.09 | 2.82±0.03 |
| + Phentolamine 10µM *(n=6)* | 2.10±0.33* | 2.33±0.52*** | 2.28±0.66* |
| + KH | 0.56±0.12* | 1.91±0.01*** | 0.61±0.47 |
| + U-73122 10µM *(n=7)* | 1.57±0.23* | 1.86±0.08*** | 0.99±0.10** |
| + KH | 0.53±0.15* | 2.11±0.05*** | 1.69±0.04*** |
| + Heparin 900UI/ml *(n=8)* | 0.51±0.07** | 2.24±0.04*** | 1.19±0.42* |

**Table 2:** Different potencies of the leaves aqueous extract of *Bidens pilosa* in presence of some modulators of the phospholipase C signaling pathway, on the contractile activity of the rat primed-estrogenized myometrium.

| *B. pilosa* Aqueous Extract | Potency (%) | | |
|---|---|---|---|
| | Amplitude | Tonus | Rate |
| + Tétrodotoxin 10µM | -8.56↓ | -8.44↓ | -111.75↓ |
| + Atropine 10µM | -38.93↓ | 231.26 ↑ | -74.51↓ |
| + Phenylephrine 10µM | -78.24*↓ | -14.36*↓ | 155.87*↑ |
| + Phentolamine 10µM | -537.66*↓ | -34.42*↓ | -185.70*↓ |
| + U-73122 10µM | -35.48↓* | -29.50*↓ | -15.72↓ |
| + Heparin 900UI/ml | -26.03↓ | -22.21↓ | -32.43↓ |

↓= Inhibition ↑=Potentialization

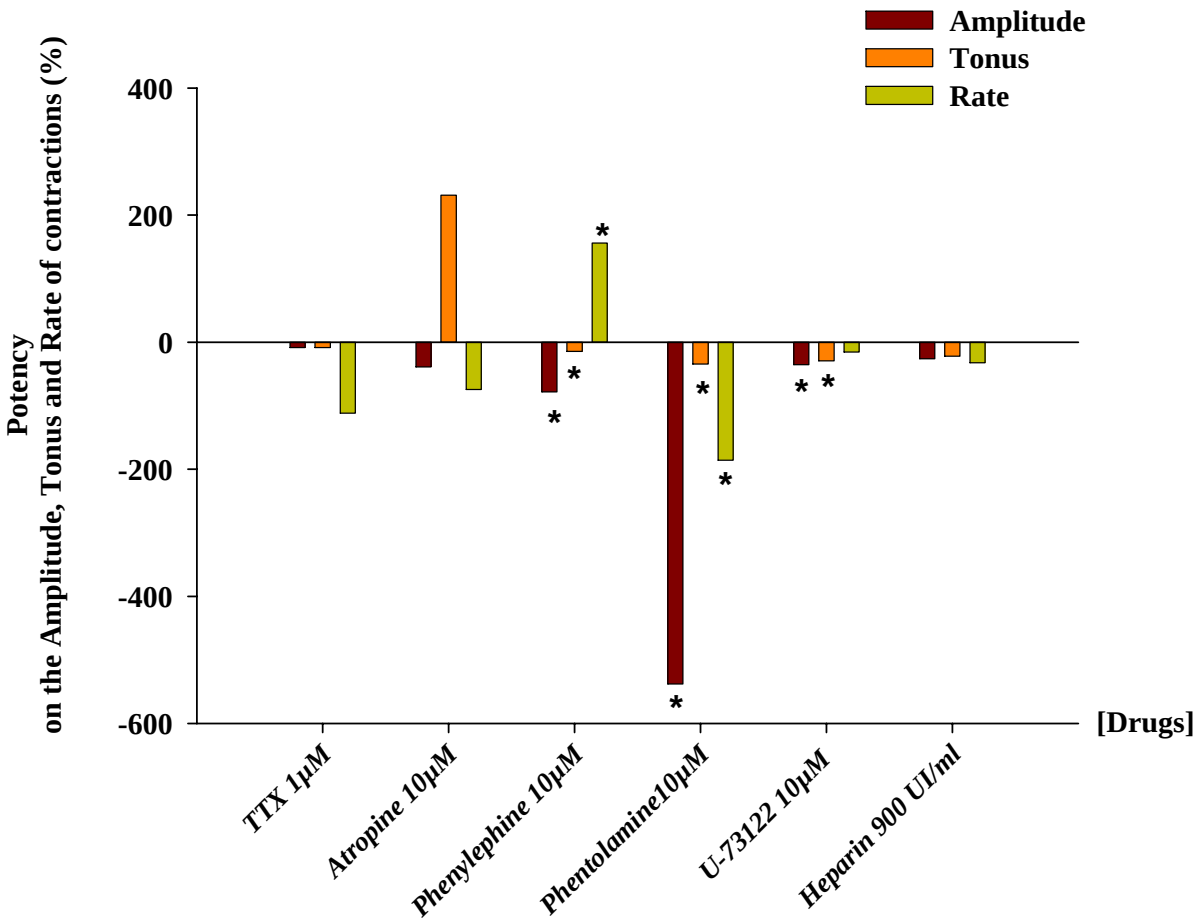


**Figure 3:** Potencies (%) of the effect of the *B. pilosa* leaves aqueous extract on the Amplitude, tonus and the rate of the contractile activity of primed-estrogenized rat myometrium, in presence of 1µm Tetrodotoxin, 10µM Atropine, 10µM Phentolamine, 10µM Phenylephrine, 10µM U-73122 and 900UI/ml Heparin.

## Discussion

During our first screening experiments, we observed that *B. pilosa* leaves aqueous extract exhibited *in vivo* uterotrophic/estrogenic and *in vitro* oxytocic/uterotonic effects on immature mouse and primed-oestrogenized rat uterine muscle *[F. Longo et al.,(2008). In vivo and in vitro effects of Bidens pilosa l. (asteraceae) leaf aqueous and ethanol extracts on primed-oestrogenized rat uterine muscle". Accepted in Afr. J. of Trad. CAM. N°239-1146-1]*. Tested in presence of the main uterine membrane modulators, *B. pilosa* aqueous extract did neither significantly (P>.05) potentiate nor inhibit the maximal effect (activation/inhibition respectively) induced by 1µM Oxytocin (a Gq/11/PLCβ receptor agonist), 60mM KCL (a potent membrane depolarizer) and 10µM Verapamil (a potent L-type Voltage-gated Ca2+ channels antagonist) on the primed-oestrogenized rat uterine contractile activity. These results have demonstrated that *B .pilosa* induce enhancement of the myometrium contractile activity occurs mainly through activation of L-type voltage gated $Ca^{2+}$ channels after binding to a protein Gαq/11 membrane receptor *[Longo et al., "In Vitro Effects of Aqueous Leaf Extract of Bidens Pilosa L. (Asteraceae) on the contractile activity of Primed-Oestrogenized Rat Uterine Strips, in Presence of Some Uterine Contractile Agents". In submission J.Ethnopharmacol. N°JEP-D-07-00588]*.

**Effects in Presence of a Sodium Channel Blocker: Tetrodotoxin**

As previously observed (23, 24) and confirmed in our experiment, TTX did not modify t(P>0.05) the uterine contractile activity. TTX is a virulent poison produced by pufferfish which inhibits nerve influx transmission at synapses by blocking selectively $Na^+$ channels on the presynaptic membrane (17). The uterine smooth muscle (post junctional membrane) also has TTX sensitive L-type $Na^+$ channels with a density increase as gestation going at term probably due to the nerve growth factor (NGF) (2). The measurement of various capacitative influx on the myometrial cell using patch clamp technique has demonstrated that these $Na^+$ channels are involved in the generation of the action potential (25) during excitation by agonists. The general weak inhibitory effect induce by TTX on the three parameters could mean a depolarizing effect of *B. pilosa* extract on the nerves endings via $Na^+$ channels with therefore, induce Acetylcholine discharge on the junctional space, bind to uterine muscarinic receptors and then induce contraction. On the other hand, previous studies have reported that the uterine muscle activity is exclusively myogenic (1) and during contraction, the $[Ca^{2+}]i$ increase is not dependant to the TTX sensitive $Na^+$ influx so, $Na^+$ channels does not participate to the agonist induce contractile activity (23, 26). Phillippe & Masa (1999) have previously concluded that the inhibitory effect of an agonist in presence of TTX is due to its poisonous presence in the experimental milieu instead of its inhibitory activity on $Na^+$ channels as there are weakly sensitive to the TTX (23). But with reference to the significant ($P \leq 0.05$), efficacy of *B. pilosa* on the amplitude and tonus, we can conclude that TTX sensitive $Na^+$ channels participate to *B. pilosa* leaf aqueous extract induced phasic and tonic components of the contractile uterine activity, at potencies of 8.56% and 8.44% respectively [Table 2; Figure3].

**Effects on the Amplitude of Contractions**

Wray et al (2003) have reported that changes in $Ca^{2+}$ signals within the myometrium have important functional consequences as they determine contractility and, the basic phasic nature of uterine contractions, essential for successful labour is critically dependent on $Ca^{2+}$ influx through voltage-gated L-type $Ca^{2+}$ channels and hence in turn on membrane potential (27). It has also been reported that protein $G_{\alpha q/11}$ receptor's agonists induced enhancement of the amplitude of contractions of smooth muscle after depolarization of the cell membrane L-type voltage gated $Ca^{2+}$ channels (VOCL), by the $IP_3$ puffs $Ca^{2+}$ coming from the sarcoplasmic reticulum (7, 8, 28). Our results have showed an inhibitory effect in presence of all protein $G_{\alpha q/11}$ coupled to receptor modulators tested on the amplitude of contractions, with a highest effect observed in presence of 10µM Phentolamine (-537.66%) [Table 2, Figure 3]. This effect was also confirmed with the high lost of *B. pilosa* efficacy in presence of this specific α1-adrenergic inhibitor and the high efficacy observed in presence of it specific agonist PHE. The variation of amplitude of contractions depends of the flux of free $Ca^{2+}$ ions entry through membrane voltage gated $Ca^{2+}$ channels (VOCLs) and α1-adrenergic receptors have been shown to induce uterine contractility via this $Ca^{2+}$ influx (29, 30). The competitive behaviour, the high efficacies and the high potencies observed in presence of α1-adrenergic receptors modulators, comparatively to other protein $G_{\alpha q/11}$ receptor inhibitors, implied that, *B. pilosa* induce amplitude increase mainly via α1-adrenergic receptors activation. Earlier studies on swine renal arteries (20) and on postpartum rat model (30) have reported that the initial transient spike of the PHE-induced calcium signal is due to release of calcium from inositol 1,4,5-trisphosphate-sensitive calcium stores evoked by alpha 1-adrenoceptor-coupled stimulation of PLC and that the maintained component is due to capacitative calcium entry,

which is modulated by protein kinases; the potentialization effect observed in presence of Phenylephrine could be due to the enhance activation of these mechanisms by *B. pilosa.*

**Effect on the Tonus:**

Contrary to the results observed on the amplitude, all tested drugs presented a weak inhibition on the tonic component of *B. pilosa* induced contractile activity. The highest inhibitory effect was observed in presence of phentolamine (-34.42%) followed by the PLC inhibitor [U-73122] (-29.50%) and the $InsP_3$-R inhibitor heparin [Table 2, Figure 3]. Earlier research works have demonstrated that the controlling muscle tonus during agonist stimulation is under the sarcoplasmic activity. The cytosol [$Ca^{2+}$] enhance the release of $Ca^{2+}$ sparks from the SR $Ca^{2+}$ induced $Ca^{2+}$ release (CICR) which then, activated capacitative influx through store operated $Ca^{2+}$ channels (SOCC) and increase muscle tonus (31, 32). Thus, the inhibitory effect observed on the tonus may be via inhibition of this pathway. Potentializations observed especially in presence of Atropine, PLC inhibitor U-73122 and phentolamine implied other signaling pathways activation which increases the tonus level. The weak potentialization (+8.45%) observed at high extract concentrations in presence of Heparin could express the limit of interference of the SR during the contractile induced activity by *B. pilosa*.

**Effects on the Rate of Contractions**

The oscillatory activity of phasic muscle is under the cycling coupling between the contractile proteins: actin and myosin. This coupling depends on the activation of a specific enzyme myosin light chain kinase (MLCK) by an activated quaternary complex: $4Ca^{2+}$-Calmodulin (6, 33). Recent studies on isolated cells using the fluorescent dye Fluo3 have showed that, calmodulin is constitutively associated with the $IP_3$-R and functions as an essential subunit for proper functioning of the $InsP_3$-R (34); thus the $InsP_3$-R activity is linked to that of Calmodulin and regulates the uterine contractile activity. The comparative potencies of *B. pilosa* aqueous extract on the oscillatory activity presented a highest inhibitory effect in presence of the membrane receptors antagonist rather than in presence of the PLC and $InsP_3$-R inhibitors. Phentolamine and Phenylephrine both α1-adrenergic modulators exhibited the highest inhibitory (-183.70%) and the potentialization (+155.87%) [Table 2, Figure 3] effects on the oscillatory activity, and once again, showed the high implication of α1-adrenergic receptors during *B. pilosa* induce rat uterine contractility.

Previous research have been reported that activation of Protein $G_{\alpha q/11}$ in addition to activation of PLC activate the cytosolic RhoGEF enzyme with phosphorylate a membrane substrate RhoA-GDP then the enzyme Rho kinase; which could activate directly or indirectly (inhibition of myosin light chain phosphatae MLCP) the myosin light chain (MLC) (35, 36). U-73122 a potent inhibitor of agonist-induced Phospholipace C activation in smooth muscle was demonstrated in porcine myometrium to inhibit the hydrolysis of $PIP_2$ to $PI_3$ up to 50% by inhibiting the coupling of the protein $G_{\alpha q/11}$ to the phospholipase $C_\beta$ activation which leads at least to a decrease of [$Ca^{2+}$]I (37-39); it has also been reported that PLC inhibition prevent $InsP_3$ formation, diminish but not abolish $Ca^{2+}$ release by sarcoplasmic reticulum stores (39, 40) and that, protein $G_{\alpha q/11}$ binding site on it regulatory subunit can be activated by many other protein kinases such as PKA, PKC and PKG (41). As oxytocic receptors, activation of α1-adrenergic receptors, protein $G_{\alpha q/11}$ coupled receptors, by *B. pilosa* could also activate various signaling pathways in the uterine cell (2, 42) such as those activated by Protein $G_{\alpha q/11}$ in addition of the PLC pathway, which likewise, enhances the contractile activity of cell by acting directly or indirectly on the myosin light chain (MLC) (6). MLCK is known to be the

ultimate key enzyme of the smooth muscle contraction and it can be activated by a direct phosphorylation of $MLC_{20}$ by Rho kinase, a $Ca^{2+}$-independent MLCK (ZIP kinase), or by an inhibition at the MYPT1/PP1C sites by Rho kinase and the phosphorylated CPI-17 respectively; MLCK is also known to contain several consensus phosphorylation binding sites outside of the catalytic core. So, the induced oscillatory contractile effect by *B. pilosa* extract may be due by such activations (6, 9).

On the other hand, *B. pilosa* is a crude extract composed with various active compounds mainly flavonoïds and polyacetylenes, and it have been reported that these compounds are responsible to *B. pilosa* biological effect (43-45). The behavioural variation induce by the extract on regard to the amplitude, tonus and the rate of contractions, could be due to one or several active molecules of the extract, which presented different acute effects on the uterine signaling pathways involvedduring the oxytocic-like contractile activation. Comparatively to Oxytocin and prostaglandins, natural utero-spasmodic substances could exert their activation through other signaling pathways like activation of capacitative calcium channels on the cell membrane (46, 47), various cytosolic protein kinases and phosphodiesterases (20) or by inhibiting cellular mechanism responsible for the $Ca^{2+}$ extrusion during relaxation (15). Experiments are going on to investigate such possibilities by antagonizing the aqueous leaf *B. pilosa* extract in presence of their specific modulators.

## Conclusion

With reference to effects of endogen hormones (Oxytocin, Prostagladins) and neurotransmitters (phenylephrine, acetylcholine) on a pregnant myometrium membrane receptors (41), our results clearly demonstrated that *B. pilosa* induced contractile effect on the uterine muscle further to activation of the $G\alpha_{q/11}$/PLCβ signaling pathway essentially via α1-adrenergic modulators and confirm the adrenergic activity of *B. pilosa* leaves extract observed on the relaxation of Norepinephrine and KCL-induced rat aortic strips by (10-12).

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