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UTERINE EFFECTS OF THE ETHYL ACETATE EXTRACT OF THE LEAVES OF *BIDENS PILOSA* (ASTERACEAE)

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ABSTRACT

The evaluation of the estrogenic (Uterotrophic and uterotonic) effect of the ethyl acetate extract of the leaf of *Bidens pilosa* was performed on immature and pregnant female rats at the doses of 625mg/kg; 1250mg/kg and 2500mg/kg *per os*, once a day, from the 18th to the 20th day after birth. The control group received Tween80 (1%), and the reference group received estradiol 1 mg/kg valeriate estradiol). The extract provoked an increase ($p<0.05$) on the wet uterine and ovaries weight at all doses, with the highest effect observed at the dose 1250mg/kg. The uterine horn's morphology and the tissue architecture was remodeled (vs. estradiol 1mg/kg) and the ovarian cholesterol level decrease ($p<0.001$) in a dose dependant manner. The uterotrophic effects were evaluated on the morphology and the relative wet weight of uterine horns and ovaries, as well as the total level of ovarian cholesterol. Furthermore, uterotonic effects were evaluated after 7 days treatment on pregnant rats which received the extract at same doses, from the 9th to the 15th day of pregnancy. Effects were evaluated on the body weight gain, pregnancy duration, viability index and weaning index, till the six living weeks of offspring. *Bidens pilosa* induced a decrease

($p<0.001$) in body weight gain and a shortening of the pregnancy duration. At the dose 625mg/kg, pregnant rats reached the term of pregnancy and delivered living offspring, while 1250mg/kg caused stillborns delivery. At the dose 2500mg/kg, *B.pilosa* induced abortion on the 18th day of pregnancy. Furthermore, the phytochemical analysis of the tested fraction using HPLC-MS techniques revealed the presence of several compounds including two major compounds and known bioactive flavonoids, namely Iso-Okanin 7-O-β-D-(2″,4″,6″-triacetyl)-glycopyranoside and Okanin 4'-O-β-D-(4″,6″-diacetyl)-glucopyranoside, which could be responsible for this estrogenic effect.

KEYWORDS*:* *Bidens pilosa*, estrogenic activity, ethyl acetate fraction, rats.

INTRODUCTION

Over the years, medicinal plants have increasingly been used in traditional medicine. In Cameroon, according to a report of the Secretary of State for Public Health, on 100 000 live births, 669 women die during childbirth.[1] Those deaths are largely due to the inability of the uterus to contract and expel the foetus during labour, constituting a danger for mother and child.[2] *Bidens pilosa* Linné is a medicinal herb belonging to the family of Asteraceae and widely distributed in areas of Africa, Asia, and tropical America regions. All parts of the plant are used, in association or not, to treat various diseases: its roots, leaves and seeds are reported to have antibacterial, antidysenteric, anti-inflammatory, antimicrobial, antimalarial, diuretic, hepatoprotective and hypotensive properties. In Africa, *Bidens pilosa* is used to treat headaches, ear infections, hangovers, diarrhea, kidney problems, malaria, jaundice, dysentery, burns, arthritis, ulcers, and abdominal problems. It is also used as anesthetic and coagulant and to ease childbirth. In sub-Saharan Africa, its fresh or dried shoots and young leaves are eaten as a leaf vegetable, especially in times of food scarcity[3] Recent scientific researches revealed some of its properties: antivenom potentiator,[4] antihypertensive;[5, 6] and oxytocic-like effect on the uterine muscle.[7-9]

Nowadays researches are focused on natural phytoestrogens especially on flavonoids due to their structural similarity with estrogenic steroids presents in the human body. On the other hand, it has been demonstrated that, natural products are benefits for women in alleviating menopausal health concerns, reduce cardiovascular diseases and breast and uterine cancers, since synthetic estrogens are known to cause endometrial and breast cancer and other adverse effects.[10, 11]

Our preliminary *in vivo* and *in vitro* studies on the aqueous extract of *Bidens pilosa* leaves has justified its empirical use as a labour facilitator during childbirth, mainly through activation of receptors coupled to Gαq proteins and activation of reticular Ca^{2+} release.[7-9] Two fractions were obtained from the partition of the crude CH_2Cl_2/MeOH extract between water and ethyl acetate. The present work aims to investigate, in details, the respective estrogenic (uterotrophic and uterotonic) effect of the ethyl acetate fraction.

MATERIAL AND METHODS

2.1. Plant material

Fresh leaves of *Bidens pilosa*, collected in Yaounde (Center Cameroon) in December 2011, were dried in the shade inside the Laboratory at room temperature, and then pulverized. The plant specimen, identified by Mr Tadjouteu Fulbert, botanist at the Cameroun National herbarium, was deposited in the database with the number Longo2 N°65656/HNC (codes bars: 4A0060204 & 4A0060205).

2.2. Animals

Female, adult *Mus musculus* mice and female immature and adults Wistar rats were used. They were daily fed and received water *ad libitum.* Animals were treated following the International ethical conduct of animal's treatment.[12-13]

2.3. Experimental procedures

2.3.1. Plant extraction

Extraction of 516g was performed with the mixture of CH_2Cl_2/MeOH (1:1) (2 x 2.5L) for 48H at room temperature. After filtration and concentration under reduced pressure on a rotary evaporator-type HEIDOLPH W2000; 60g of the crude extract were obtained. Part of this extract (55g) was re-extracted with 100mL of ethyl acetate to afforded 26.7g of the ethyl acetate extract named BpEA (yield of 5.17%). For experimentation, the extract was diluted in Tween80 (1%) for its nontoxic effect and for it lipids dilution property. The plant treatment was conducted as recommended.[14]

2.3.2. Chromatographic analysis

The high resolution mass spectra were obtained with an LTQ-Orbitrap Spectrometer (Thermo Fisher, USA) equipped with HES I-II source. The analysis was performed by using a Nucleodur Gravity column (50 x 2 mm, 1.8µm particle size) from Macherey-Nagel (Düren, Germany) with a H_2O (+ 0.1 % HCOOH, + 10mM NH_4Ac) (A) / acetonitrile (+ 0.1 %

HCOOH) (B) gradient (flow rate 300 µL/min). Samples were analyzed by using a gradient program as follows: 90% A isocratic for 2 min, linear gradient to 100 % B over 13min, after 100 % B isocratic for 5min, the system returned to its initial condition (90% A) within 0.5min, and was equilibrated for 4.5min. Chemical structures were drawn using the CS Chemdraw ultra program.[15]

2.3.3. Acute toxicity essay

Two groups of five female mice orally received an acute dose of 2g/kg of *Bidens pilosa* ethyl acetate extract. They were observed during seventy two hours and the number of dead mice was recorded.[16]

2.3.4. Three days' uterotrophic essay on immature female rats

Twenty five (25) rats aged 18 days and weighting about 30 g were used. After early weaning on postnatal day 18, they were grouped in 2 or 3 per cage and fed once a day during three consecutive days from day 19 to Day 21 at 8 am ± 15 min, using an oesophageal catheter. Rats were divided into 4 groups: two groups received 625mg/kg and 1250mg/kg of BpEA, one reference group received estradiol valerate (1mg/kg) (Progynova® 2mg) and the fourth group received Tween80 (1%). Twenty four hours after Day 21, rats were euthanized by ether inhalation in a closed environment for necropsy.[17] Uterine horns and ovaries were gently removed and cleaned of fats and mesenteries. Ovaries were dissected and weighted.[18, 19] One uterine horn and ovaries of each rat was separately ground (on an ice tray) and homogenised at 20 % in a 25mM Tris-HCl buffer, pH 7.5, stored at -20°C in a 5mL tube for total cholesterol analysis. Uterine horns were weighted (Care was taken to avoid loss of intrauterine fluid) and preserved in 10% neutral Buffered formalin for tissue cross-section.

2.3.4.1. Histological analysis

Histological cross sections were performed by steps.[20] Briefly, samples were successively dehydrated in ethanol (from 70% - 100%). Then, paraffin embedding was applied followed by a serial sectioning of samples less than 7µm thickness. Sections were stained with hematoxylin and eosin, according to standard procedures for selected slides for analyzing muscle/connective tissue composition and elastic fiber pattern. Cross sections were convert to slides into digital pictures (x100 or x400) using a Sony Camera.

2.3.4.2. Determination of the ovarian total cholesterol

The ovarian total cholesterol level was determined using the direct LDL Cholesterol Liquid Reagent kit (SGMitalia Diagnostics, Rome, Italy). Under the action of cholesterol esterase, cholesterol is converted into esterified cholesterol and fatty acids; its oxidation in the presence of cholesterol oxidase produced cholesterol-3-one and hydrogen peroxide; but, in the presence of the catalytic action of peroxidase, it produced 4-aminoantipyrine and phenol. The absorbency of the samples (Ech Abs) and that of the standard (Std. Abs) were read at a wave length comprise between 570-600 nm; against blank after 60 min. The total cholesterol content in the samples was calculated using the formula: Total Cholesterol | ((mg / dL)) = Abs sample / Abs standard × standard concentration; *Standard concentration = 200 mg / dL; Abs = absorbance.*

2.3.5. Uterotonic essay on pregnant rats

Pregnant rats aged 10 to 14 weeks and weighting between 170-200g, were divided into 3 groups of 5 rats each: the two tested groups received BpEA 625mg/kg and 1250mg/kg, and the normal control group received Tween 80 (1%). Before the experiment, the oestrous or pro-oestrous stages were detected by observing vaginal smear under microscope (Type Optic Ivymen System) then, they were mated with males with a mating index of one male/ two females, around 6 pm ± 15 min.

2.3.5.1. Analysis of vaginal smear

Vaginal secretions were removed after 2 to 3 enemas, using a Pasteur pipette filled with 1mL NaCl 9‰. The smear was then collected and gently spread on a glass slide and placed to dry at room temperature. After being fixed in methanol (95%) for 2 to 3min then with May-Grumwald for 2-3min and Giemsa for the same time; slides were observed under optical microscope (x30). The presence or corneous vaginal epithelium cells determine the stage of the oestrous cycle. Only rats at oestrous stage were crossed with males. The presence of sperm in the vaginal smear indicated mating, and that day was considered to be Day1 of pregnancy.[8, 21]

2.3.5.2. Experimentation

Rats were fed from the day 9th to the 15th day of pregnancy as described on top. After the 15t^{h} day of feeding, rats were observed daily until parturition. The body weight, pregnancy duration, viability (offspring's number/litter) and weaning indexes and offspring body weight variation during 6 weeks were recorded. Let's notice that, from the 7th to the 8th day of

pregnancy, before the beginning of feedings, vaginal smears were collected again and the observation of an oestrus cycle blocked at proestrus1 stage, confirmed the efficiency of the pregnancy.

2.4. Chemicals

NaCl 9‰; Estradiol valerate (Progynova 2mg, Lot N° 11116A Delpharm, Lille; Buffer solution (sodium phosphate) pH 7.3; (Solution 1: 1.175 g NaH_2PO_4, 4.4 g NaCl and 5.8 g Na_2HPO_4 + Solution 2: 0.5g of gelatine (pH 7.3 with 1M NaOH); Tween80: Lot 90H0678.

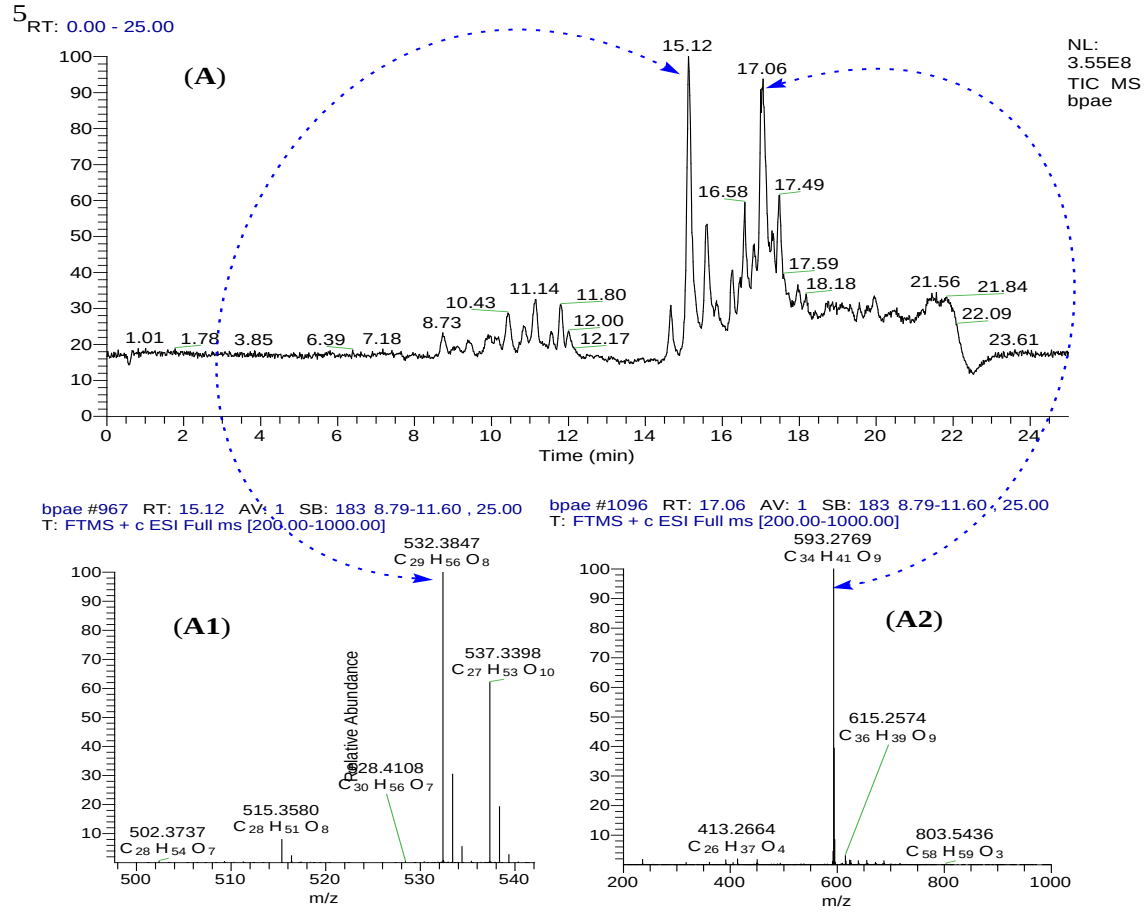
2.5. Statistical Analysis

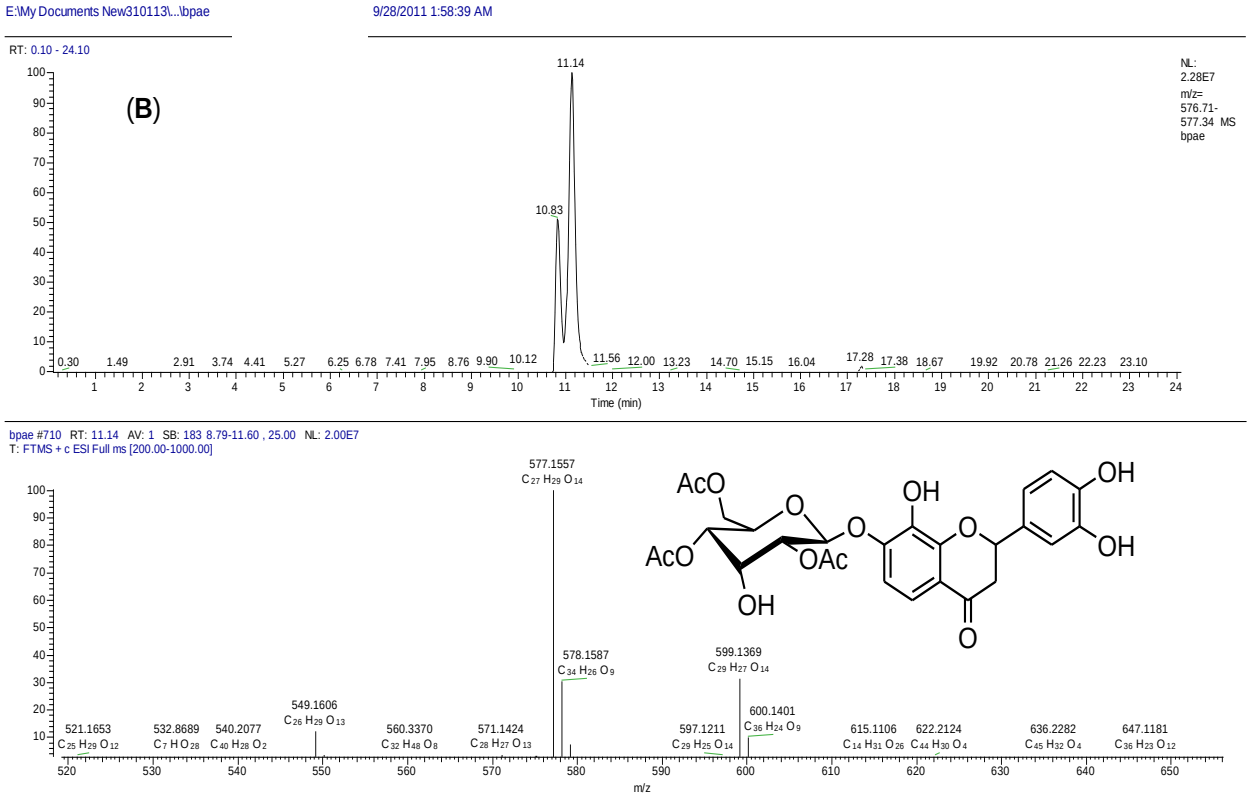
Statistical analyses were done using Sigma 2.03. Results were expressed as mean ± S.E.M. The group comparison was done using the parametric test of variance analysis of One-way ANOVA followed by non-parametric tests "t" of Student, Tukey, Dunnett or Mann-Whitney, according to results. The difference between two sets of values was considered significant at $p < 0.05$.

RESULTS

3.1. HPLC analysis

The HPLC technique with the mass spectrometer revealed, after 25min, a chromatographic profile with two main peaks (Fig. 1A): (A1) HR-MS $[M+H]^+$ *m/z* $[M+H]^+$: *m/z*; (calcd. for $C_{29}H_{56}O_8$; 532.3846, RT 15.11, and (A2) HR-MS $[M+H]^+$*m/z* $[M+H]^+$: *m/z*; (calcd. for $C_{30}H_{49}O_8$; 537.3403; RT 17.04). In addition, two peaks were observed corresponding to two known flavonoids [23]: a flavonol: HR-MS $[M+H]^+$*m/z* 577.1557 (calcd. for $C_{27}H_{29}O_{14}$ 577.1557 (Iso-Okanin7-O-β-D-(2",4",6"-triacetyl)-glycopyranoside), with RT 11.14 min (Fig. 1B), and a Chalcone: HR-MS $[M+H]^+$ *m/z* 535.1448 (calcd. for $C_{25}H_{27}O_{13}$, 535.1452) (Okanin 4'-O-β-D-(4",6"-diacetyl)-glucopyranoside), RT 10.43 min (Fig. 1C).


RT: 0.00 - 25.00
(A)
NL: 3.55E8 TIC MS bpae
Time (min)
bpae #967 RT: 15.12 AV: 1 SB: 183 8.79-11.60 , 25.00
T: FTMS + c ESI Full ms [200.00-1000.00]
(A1)
532.3847 C29 H56 O8
537.3398 C27 H53 O10
528.4108 C30 H56 O7
515.3580 C28 H51 O8
502.3737 C28 H54 O7
Relative Abundance
m/z
bpae #1096 RT: 17.06 AV: 1 SB: 183 8.79-11.60 , 25.00
T: FTMS + c ESI Full ms [200.00-1000.00]
(A2)
593.2769 C34 H41 O9
615.2574 C36 H39 O9
413.2664 C26 H37 O4
803.5436 C58 H59 O3
m/z


E:\My Documents New310113\...\bpae
9/28/2011 1:58:39 AM
RT: 0.10 - 24.10
(B)
NL: 2.28E7 m/z= 576.71-577.34 MS bpae
Time (min)
bpae #710 RT: 11.14 AV: 1 SB: 183 8.79-11.60 , 25.00 NL: 2.00E7
T: FTMS + c ESI Full ms [200.00-1000.00]
577.1557 C27 H29 O14
578.1587 C34 H26 O9
599.1369 C29 H27 O14
600.1401 C36 H24 O9
549.1606 C26 H29 O13
521.1653 C25 H29 O12
532.8689 C7 H O28
540.2077 C40 H28 O2
560.3370 C32 H48 O8
571.1424 C28 H27 O13
597.1211 C29 H25 O14
615.1106 C14 H31 O26
622.2124 C44 H30 O4
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647.1181 C36 H23 O12
AcO
OAc
OH
O
m/z

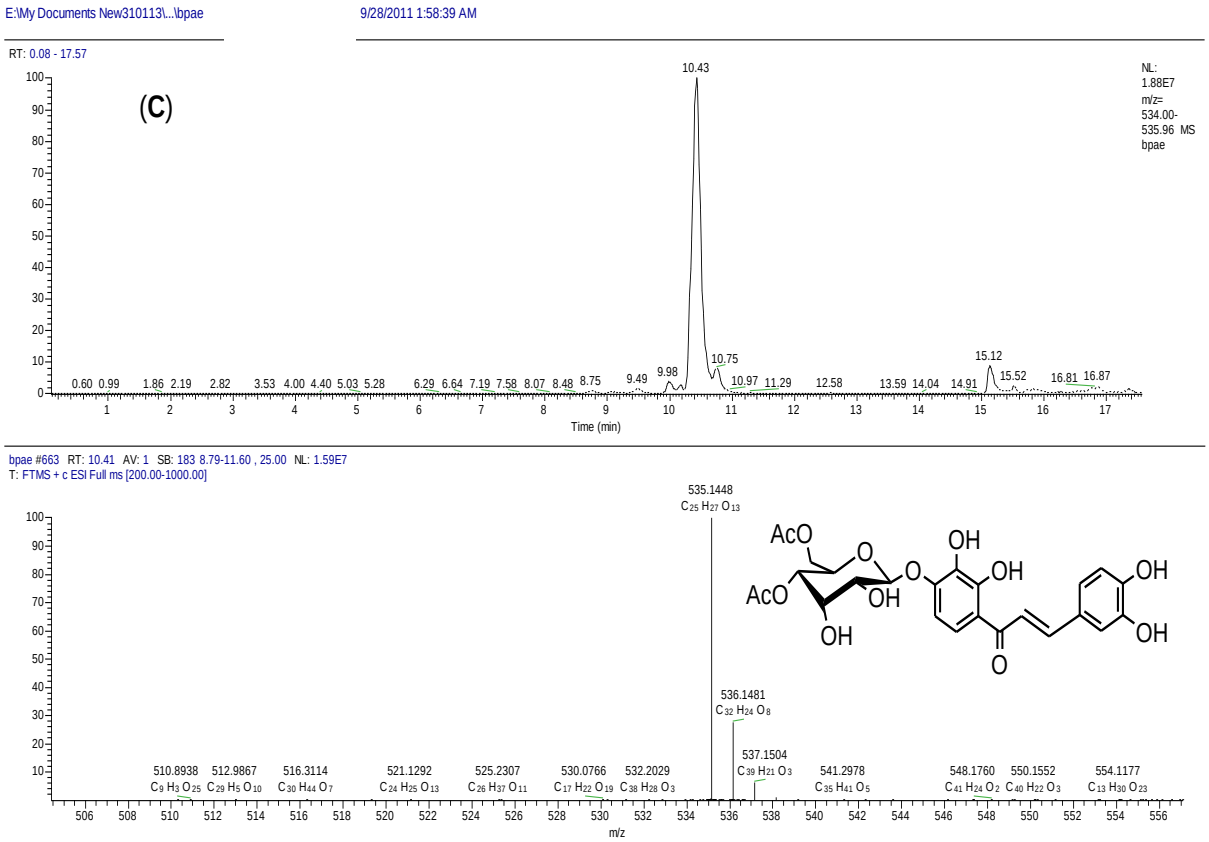

Figure 1: LC/MS profile chromatogram for: (A) the crude ethyl acetate extract of *Bidens pilosa*.; and the two major compounds [RT 15.11, *m/z*, $C_{29}H_{56}O_8$; $[M+H]^+$= 532.3846; RT 17.04, *m/z*, $C_{30}H_{49}O_8$; $[M+H]^+$= 593.2769 (B) Iso-Okanin7-O-*β*-D-(2",4",6"-triacetyl)-glycopyranoside RT 11.14mn, HRMS $[M+H]^+$*m/z* 577.1557 (calcd. for $C_{27}H_{29}O_{14}$; 577.1557and (C) Chalcone Okanin 4'-O-*β*-D-(4",6"-diacetyl)-glucopyranoside, RT 10.43 mn, HRMS $[M+H]^+$ *m/z* 535.1448 (calcd. for $C_{25}H_{27}O_{13}$, 535.1452).

3.2. Acute toxicity

Acute administration of BpEA (2000mg/kg) did not kill mice nor modify their behaviour during the three experimental days, till one week after.

3.3. Uterotrophic effect of BpEA on immature rats

3.3.1. On the uterine horns and ovaries relative wet weight

BpEA increased both the uterine and ovaries relative wet weights, at all doses:

On wet uterine horns, the dose 1250 mg / kg caused the highest increase of 0.30% while the doses of 625 mg / kg caused the value of 0.16% (vs. estradiol).

On ovaries, similarly to its effects on uterine horns, the highest increase of 0.36% $p<0.05$ was observed at the dose 1250 mg / kg (vs. control & estradiol) (Fig.2).

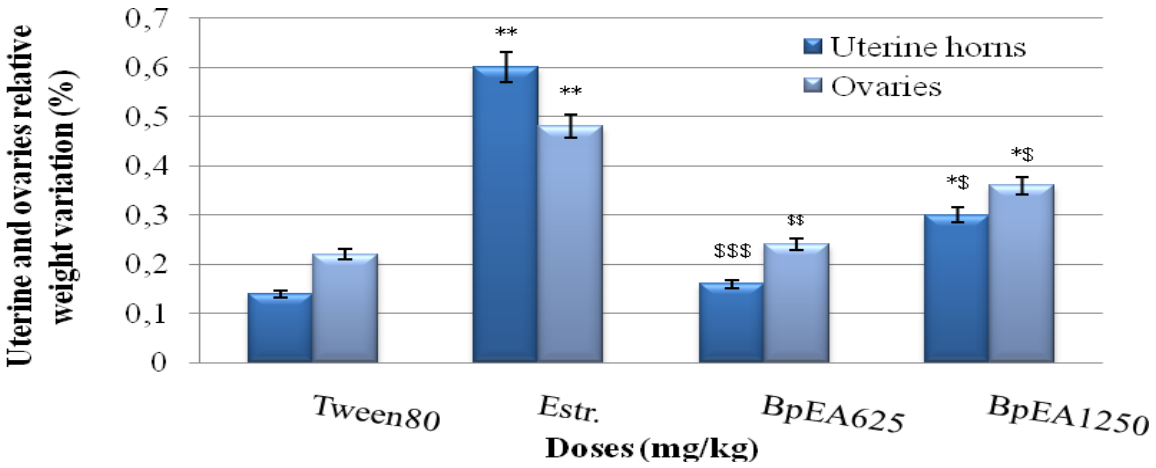

Figure 2: Effect of ethyl acetate fraction of CH_2OH/MeOH extract of *Bidens pilosa* on immature uterine horns and ovaries of female rats.

*Each bar represents mean ± SEM, n=5; *p<0. 05;**p<0.01: significant difference vs. control; §p<0. 05. §§p<0. 01; §§§ p<0.001: significant difference vs. estradiol*
CN: Control; OEst.: estradiol; BpEA: Bidens pilosa

3.3.2. On the ovarian total cholesterol level

BpEA led to a significant decrease (p<0.001) of the ovarian cholesterol level, at all doses. The highest decrease was recorded at dose 1250mg/kg, equivalent to 2.9mg/dL respectively, similarly to estradiol: 3mg / dL (Fig.3).

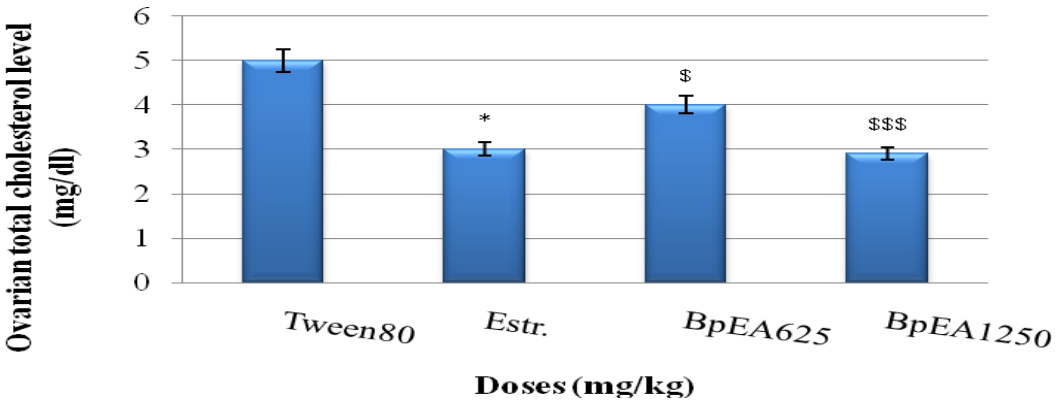

Figure 3: Effect of ethyl acetate fraction of CH_2OH/MeOH extract of *Bidens pilosa* on the ovarian total cholesterol level of immature female rats.

*Each parallel bar represents mean ± SEM, n=5; *p<0.05; significant difference vs. control; §p<0.05. §§§ p<0.001: significant difference vs. estradiol. Oest. estradiol; BpEA: Bidens pilosa*

3.2.3. On the uterine horns' tissue morphology and histology

BpEA at dose 625mg / kg *b.w.*, induced no change on the morphology of uterine horns (Fig.4$A_{1, 2, 3, 4}$), which are thin and milky colour similarly to that of the control group. At the dose 1250mg/kg *b.w.*, the uterine horns were les swollen, translucent with a liquid in their lumen, similarly to horns impregnated with estradiol (Fig.4A_4).

Cross section of uterine horns' analysis showed changes on their epithelium and lumen: BpEA 625mg/kg couldn't change the uterine epithelium and lumen which stayed identical to the control group, characterized by a thin epithelium and mucosa, and a large lumen (Fig.4B_3-C_3). At the dose 1250mg/kg, BpEA provoked a strong endometrial development, accompanied by a reduction of the lumen (Fig.4B_2 & Fig.4C_2).

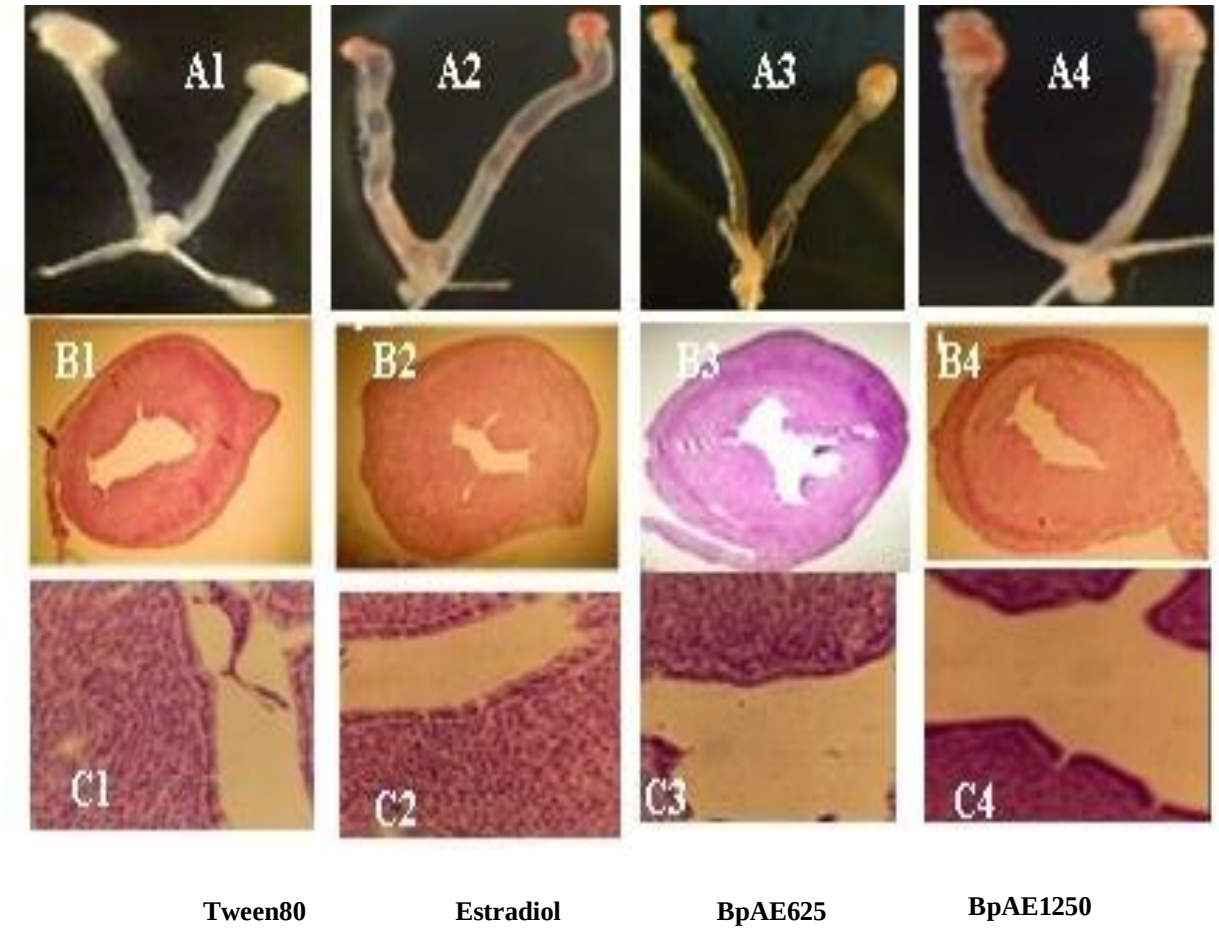

Figure4: Effect of ethyl acetate fraction of CH_2OH/MeOH extract of *Bidens pilosa* on the Dams' uterine horns morphology and histology at doses 625 and 1250mg/kg compared to normal rats (control) and estradiol.

BpEA: Bidens pilosa , X100: B_1-B_4; X400: A1-A4 & C1-C4

3.4. Uterotonic effects of BpEA on pregnant rats

3.4.1. Dam's body weight variation

During seven days, BpEA significantly ($p < 0.001$) decreased dams body weight gain up to 36% at the dose 1250mg/kg *b.w.* (Fig.5).

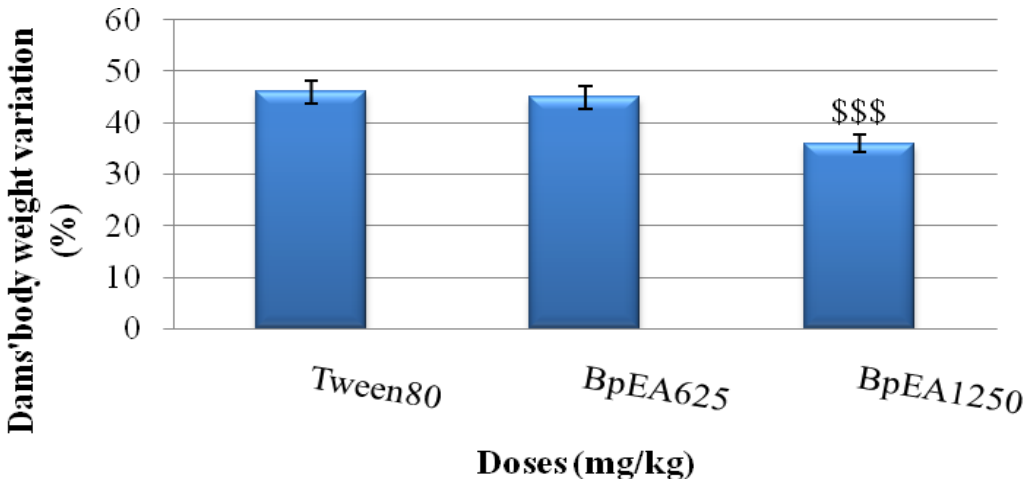

Figure 5: Effect of ethyl acetate fraction of CH_2OH/MeOH extract of *Bidens pilosa* on the rat body weight on the 9th to the 15th days of pregnancy.

C: Control; BpEA: Bidens pilosa; each bar represents mean ± SEM, n=5; $^{***}p<0,001$: significant difference vs Control

3.4.2. Effect of BpEA on the gestational duration

BpEA at dose of 625mg/kg did not ($p > 0.05$) changed the duration of gestation: all dams delivered the 22nd day of gestation similar to the control group. However, at the dose 1250 mg / kg, while non significant, dams gave birth on the 20th day ($P>0.05$).

3.4.3. Number of offspring per litter and the day survival index (viability index: VI)

BpEA induced no effects ($P>0.05$) on the 100% viability index at all tested doses: 625mg/kg and 1250mg/kg.

3.4.4. Offspring's body weight and the weaning index

Seven days administration of BpEA did not cause any change ($p>0.05$) on offspring's body weight gain (data not shown). All offspring borned in tested groups were still alive and in good health after weaning.

DISCUSSION

The *in vivo* evaluation of the estrogenic activity (uterotrophic and uterotonic) of the ethyl acetate fraction of *B. pilosa* (BpEA) showed interesting results.

BpEA is weakly toxic, according to the scale of Hodge and Sterner, as the dose 2000mg/kg[24] failed to kill mice, corresponding to a toxicity indice = 4.[24, 25]

Uterotrophic activity performed on immature rats, showed increases of uterine wet weight and uterus aqueous imbibitions, accompanied with vagina epithelial cells' cornification, characteristics of an uterotrophic effect.[26-28] The observed uterotrophic activity usually occurred during uterine morphological changes, issued from the remodelling of uterine architecture and biochemical changes[29] BpEA strongly stimulates cell proliferation in the endometrial tissue (uterotrophic activity)[21] at the dose 1250mg/kg. It has been shown that administration of estrogens or estrogenic substances increases ovary weight[19, 27] Indeed, the ovary is the seat of steroidogenesis and is responsible for the synthesis of female sex hormones[31] Similarly, the best remodelling of the uterine tissue architecture was observed at dose 1250mg / kg. Indeed, at this dose, BpEA induces a decrease in the uterine lumen and a development of uterine glands. Those effects are similar to the activity of estradiol and in accordance with previous study[21], thus demonstrates and confirmed the uterotrophic effects firstly observed with the aqueous leaf extract,[8] which was more effective at the dose 2000µg/g/day.

The estrogenic effect of BpEA was also confirmed at the biochemical level, by the decrease of the cholesterol level in ovaries of treated rats, especially at the dose 1250mg/kg ($P<0.001$). It's known that Estrogens are synthesized in the ovarian follicle cells of *theca interna* and granulose and; most of the cholesterol required for their synthesis is captured at the level of the adrenal from plasma LDL (Low Density Lipoproteins). Their biosynthesis is mainly from androstenedione, testosterone and, 16 α-hydroxytestosterone by aromatization.[31, 32] BpEA decreased ($P<0.05$) pregnant rats' body weight gain at doses 1250 and 2500mg / kg (vs. normal rats). However, it is known that during pregnancy, under the effect of estrogens, the entire organism undergoes morphological and physiological changes that result, in most cases, by a weight gain ($P<0.001$) of dams.[33] Thus, this result confirmed the slimming effect of *Bidens pilosa* leaves.[8] Our previous work on the aqueous extract demonstrated its effects on the shortening of the pregnancy duration at the dose 2000 mg/kg, when administrated during the 3rth term of pregnancy.[8] It is known that the labour can be induced by an

uterotonic effect, secondary to stimulation of oxytocin and prostaglandins secretion (hormones which are the main agonists of uterine smooth muscle), leading to a "miss-implantation" or the detachment of the embryo to the uterine wall. Also, the prostaglandins F (PGF) and E (PGE_2) secretion is generally enhanced by estrogens in pregnant women and rats.[34] This mechanism could also be one of the BpEA extract on pregnant rats.

Uterotrophic and uterotonic effects of BpEA can be explained by the nature of the compounds (flavonoids) presents in the extract.[34] From birth to day 42 (six weeks), the rats' offspring body weight didn't change ($p>0.05$) (vs. offspring from normal rats). This could mean that the extract did not modify offspring's' weight gain after birth. However, a teratogenic study is needed to confirm this.

Results also revealed the presence of bioactive molecules which could have (direct and/or indirect estrogens-like activity and improves oestrogen receptors (ER_α and ER_β predominantly).[9] Indeed, the presence of two bioactive flavonoids: 1/the Iso-Okanin-7-O-β-D-(2",4", 6"-triacetyl)-glycopyranoside and the Okanin 4'-O-β-D-(4",6"-diacetyl)-glucopyranoside, with known anti-inflammatory properties,[23] could be responsible of the phyto-estrogenic effect observed.

CONCLUSIONS

The goal of assessing the estrogenic effects of the ethyl acetate fraction of the methylene chloride/methanol extract of *B. pilosa* leaves, has led to an uterotrophic effect on the uterine horns, and an indirect uterotonic effect on pregnant rats, especially at the dose of 1250mg/kg which shortening the term of gestation to 2 days. Both effects could mainly result to the presence of bioactive flavonoids of the plant leaves. Thus, *Bidens pilosa* could be used as a natural phytoestrogen because of its uterotrophic effects. This result also confirmed the choice of traditional practitioners to use the plant leaf extract only after the beginning of labour. Furthermore, this fraction is more effective than the aqueous leaf extract effects firstly obtained, which was more effective at the dose of 2000mg/kg.

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