Full Paper

Effects of *Millettia macrophylla* (Fabaceae) Extracts on Estrogen Target Organs of Female Wistar RatStéphane Zingue^{1,2,3}, Dieudonné Njamen^{1,*}, Job Tchoumtchoua^{1,3}, Maria Halabalaki³, Evan Simpson⁴, Colin Clyne⁴, and Chantal Beatrice Magne Nde⁴¹Laboratory of Animal Physiology, Department of Animal Biology and Physiology, Faculty of Science, University of Yaounde I, P.O. Box 812 Yaounde, Cameroon²Laboratory of Physiology, Department of Life and Earth Sciences, Higher Teachers' Training College, University of Maroua, P.O. Box 55 Maroua, Cameroon³Division of Pharmacognosy and Natural Products Chemistry, Faculty of Pharmacy, University of Athens, Panepistimioupoli Zografou 15771, Athens, Greece⁴Prince Henry's Institute of Medical Research, Monash Medical Centre, 246 Clayton Road, Level 4, VIC 3168, Melbourne, Australia

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Abstract. The present study aims to determine the estrogenicity of *Millettia macrophylla*, a Cameroonian medicinal plant, in ovariectomized rats and to investigate the underlying mechanisms, in order to justify scientifically its traditional use. To accomplish this objective, we used dichloromethane (DCM) and methanol (MeOH) extracts of the stem bark of *M. macrophylla*. In the cell culture based assay, the MeOH extract significantly transactivated estrogen receptor α (ER α) and estrogen receptor β (ER β); in addition, the estrogen-like effects of both, DCM and MeOH extracts, could be inhibited in vitro by the pure ER antagonist ICI 182,780, indicating that these effects were primarily mediated through ERs. In animal experiments, both DCM and MeOH extracts significantly increased the uterine and vaginal epithelial heights in the 3-day treatment assay, while only the MeOH extract exhibited such effects in the sub-chronic treatment regimen. Furthermore, the MeOH extract significantly decreased fasting serum triglycerides, total cholesterol levels and atherogenic risk in the sub-chronic treatment. These results indicate that *M. macrophylla* extracts have estrogen-like effects supporting their traditional use in Cameroon to alleviate some menopausal problems (See graphical abstract in Supplementary Fig. 1, available in the online version only).

[Supplementary Figures: available only at <http://dx.doi.org/10.1254/jphs.13094FP>]

Keywords: *Millettia macrophylla*, female reproductive health, estrogen-like effect, estrogen target organ, phytoestrogen

Introduction

Estrogens are steroid hormones integral to the human endocrine system produced principally by the ovaries from puberty. They regulate the growth and development of reproductive organs as well as the homeostasis of a variety of tissues (1). In climacteric and postmenopausal

women, low serum levels of 17 β -estradiol often result in symptoms such as hot flashes, genital-urinary atrophy, or degenerative processes such as osteoporosis (2). Over decades, hormone replacement therapy (HRT) has been successfully used to alleviate these problems (3), although large clinical trials have mentioned serious side effects (4, 5). This has resulted in a search for HRT alternatives, and plant-derived substances, so-called phytoestrogens, are being considered (6). In most developing countries, 80% of the population still resort to traditional medicine for their primary health care (7). For

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instance, the use of traditional herbal medicine is popular in Central Africa particularly in Cameroon mainly because of economic constraints and the strength of traditional beliefs (8, 9). The limited scientific evidence about traditional medicine / complementary and alternative medicine's (TM/CAM's) safety and efficacy as well as other considerations have led the Cameroon Government in collaboration with the World Health Organisation (WHO) to put in place a strategic platform for the practice and development of TM (10). *M. Macrophylla* also known as *Millettia aboensis* is one of such medicinal plants. It is popular in many Sub-Saharan African countries including Cameroon, where different parts (stems, leaves, roots) are used as concoctions (generally prepared by mixing about 100 g of the plant in 1.5 liters of water for 15 min) or macerations (generally prepared by mixing about 1 kg of the plant in 5 liters of palm wine for 2 days) to treat various ailments including respiratory difficulties, constipation, colds and headaches, and jaundice (11, 12). *M. Macrophylla* is also used to alleviate venereal diseases as well as some symptoms related to menopausal complaints like other species of *Millettia* genus such as *M. conraui*, *M. drastica*, *M. griffoniana*, *M. zechiana*, and *M. barteri* (11, 13 – 16). Since there were no scientific data to support the above claims, we therefore aimed to evaluate the beneficial effects of *M. macrophylla* extracts on female reproductive health, in particular menopausal problems, in order to justify scientifically the claimed beneficial effects. To achieve our goal, both non-polar (dichloromethane soluble) and polar (methanol soluble) extracts of *M. macrophylla* were produced and their ability to transactivate estrogen receptor α (ER α) and estrogen receptor β (ER β) and to mimic estrogenic activity in ovariectomized rats were evaluated.

Materials and Methods

Plant material

Stem barks of *M. macrophylla* were collected in Kumba (South-West region of Cameroon) in January 2010 and identified at the Cameroon National Herbarium (HNC) (Voucher specimen No. 49654/HNC). The dried and ground stem bark of *M. macrophylla* (1000 g) was macerated in 5 liters of dichloromethane (DCM) for 72 h at room temperature, and 18 g of extract was obtained after evaporation of the solvent using a rotary evaporator in vacuum. The residue powder was thereafter steeped in 5 liters of methanol for 72 h (MeOH), filtered, and concentrated to give 47 g of methanol extract of *M. macrophylla*.

HPLC-DAD analysis of *Millettia macrophylla* (Fabaceae)

The LC analyses were performed on a Thermo Finnigan HPLC system consisting of a vacuum degasser, a quaternary pump, an autosampler, and a DAD, Diode-Array Detector (Thermo Finnigan, San Jose, CA, USA). A Discovery SUPELCO HS C-18 column (25 $\times$ 4.6 mm, 5- μ m particle size) was used for separations while the system was controlled by ChromQuestTM 4.1 software. The binary mobile phase consisting of 1% acetic acid (A) and methanol (B) was used. A gradient program was applied as follows: 2% – 98% B over 60 min, isocratic 98% B for 5 min, 98% – 2% B over 1 min, and isocratic 2% B for 9 min before the next injection. The injection volume was set to 20 μ L and the flow rate, to 1 mL/min. DAD conditions: 254, 280, and 365 nm and recording window from 200 to 700 nm.

Cell culture, transfections, and luciferase assays

In the first set of experiments, we tested the ability of different extracts of *M. macrophylla* to activate ER α and ER β , in cell-based assays. Human Embryonic Kidney 293T cell line (HEK293T) that contains the SV40 Large T-antigen was purchased from ATCC (The Global Bioresource Center, Sidney, Australia). HEK293T cells were transiently transfected with the estrogen α -receptor plasmid (200 ng HEK293T-ER α cells) or the estrogen β -receptor expression plasmid (200 ng HEK293T-ER β cells), together with the double estrogen response element (ERE) and a luciferase reporter [250 ng (ERE)₂-tk-Luc] plasmid kindly provided by Dr. Simon Chu (Prince Henry's Institute of Medical Research, Melbourne, Australia) and β -galactosidase reporter plasmid using Lipofectamine Reagent (Invitrogen, Mulgrave, Australia). They were then treated with different concentrations of the MeOH or DCM extracts of *M. macrophylla* alone or in combination with E2 or ICI 182,780 for 24 h. Cells treated with E2 alone served as positive control. Reporter gene assays in HEK293T-ER α cells and HEK293T-ER β cells were performed using a commercial kit (Promega, Melbourne, Australia) according to the manufacturer's instructions. Luciferase activity was measured and normalized against β -galactosidase activity determined by using the 2-nitrophenyl β -D-galactopyranoside (ONPG) method (Sigma-Aldrich, Sydney, Australia). Each experiment was performed in at least duplicate and repeated three times.

Animals

Juvenile female Wistar rats aged 10 to 12 weeks (250 g) were obtained from the breeding facility of the laboratory of Animal Physiology, University of Yaounde I (Yaounde, Cameroon). They had free access to a standard soy-free rat chow and water ad libitum. Animal

housing and experiments were carried out following the guidelines of the Institutional Ethic Committee of the Cameroon Ministry of Scientific Research and Innovation, which has adopted the guidelines established by the European Union on Animal Care (CEE Council 86/609).

Study design

Estradiol valerate (Progynova[®], 2 mg; Delpharm, Lille, France) and the extracts of *M. macrophylla* were dissolved in 10% ethanol with dH₂O. For the studies presented in this paper, three doses were used: 100, 200, and 300 mg/kg BW.

Experiment 1: 3-day treatment

Seventy female Wistar rats were submitted to bilateral oophorectomy under ketamin and valium anesthesia (respectively, 10 and 50 mg/kg BW, i.p.). After 14 days of endogenous hormonal decline (OECD, 2007), animals were randomly distributed into 14 groups of five animals each. The negative control group (Control) was treated with the vehicle (10% ethanol). The positive control group received estradiol valerate (E2V) as the standard drug at the optimal dose of 1 mg/kg BW per day. The remaining groups received either the DCM extract or the MeOH extract of *M. macrophylla* at doses of 100, 200, and 300 mg/kg BW per day for 3 days in the presence or absence of E2V. The organ collection and histological analysis were performed as described before by Njamen et al. (15) with the difference that mammary glands were collected. The uterine weight, total protein levels in uteri, uterine and vaginal epithelial heights, and mammary gland alveolar and ductal structures were assessed.

Experiment 2: sub-chronic (42-day) treatment

Thirty animals were either sham-operated or ovariectomized. Eighty-four days (12 weeks) after endogenous hormonal decline and set up of human-like postmenopausal symptoms (15), animals were randomly allocated to 6 groups of five animals each. They were treated for 42 days with vehicle (OVX and Sham groups) or estradiol valerate (E2V) (1 mg/kg BW per day), and only the doses of extract that showed a promising biological activity on the first experiment (DCM: 300 mg/kg BW per day and MeOH: 100 and 300 mg/kg BW per day). Animals were weighed once every two weeks throughout the experiment in order to determine the body weight gain. Thereafter, rats were sacrificed by decapitation. Blood samples were collected and centrifuged at 3000 g for 15 min and serums were stored at -4°C for biochemical analysis. The organs (mammary glands, vagina, and uteri) were collected for histological analysis. Fresh uteri were weighed using an electronic scale.

Histological analysis

Using the complete Zeiss equipment consisting of a microscope (Axioskop 40) connected to a computer where the image was transferred and analyzed with the MRGrab1.0 and Axio Vision 3.1 software, all provided by Zeiss (Hallbermoos, Germany) (15, 17, 18); the histomorphology of the mammary glands, as well as the uterine and vaginal epithelial heights, were assessed from 5- μ m sections of paraffin-embedded tissues following hematoxylin-eosin staining.

Biochemical analysis

Total uterine protein levels were determined in uteri using colorimetric methods described by Gonal et al. (19). Total-cholesterol, HDL-cholesterol, and triglycerides blood levels were determined enzymatically using reagent-kits purchased from Biolabo (France). LDL-cholesterol was estimated from total-cholesterol, HDL-cholesterol, and triglycerides by using the formula of Friedewald et al. (20).

Statistical analysis

The data from each experimental group were expressed as the mean $\pm$ S.E.M. One-way analysis of the variance (ANOVA) followed by Dunnett's test for multiple comparisons and the Student *t*-test were used for statistical comparison between different control and treated groups for in vivo and in vitro experiments, respectively. The significance of the difference was fixed at $P < 0.05$.

Results

Phytochemical analysis

The HPLC-DAD chromatograms revealed the differences in the chemical profiles of the DCM and the MeOH extracts. Using chromatographic and spectroscopic features such as retention time and UV-Vis spectra together with in-house library comparison the main chemical classes of constituents were tentatively identified (Fig. 1).

As expected, the DCM extract is characterized by the presence of more lipophilic compounds such as terpenoids and fatty acids while flavonoids and isoflavonoids aglycons, mainly methylated, are also present but in low percentage. The MeOH extract exhibited a richer phytochemical content with components covering the whole range of polarity based on the retention time at the chromatogram. The method used allowed the identification of simple phenolics (21), flavonoid glycosides, and polar flavonoids (22) as well as methoxylated flavonoids and isoflavonoids (23) in the MeOH extract.

Transactivation assay

Estrogen receptor transactivation: Firstly, the effects

Fig. 1. Comparative HPLC chromatogram of *Millettia macrophylla* DCM and MeOH extracts and some typical UV spectra at 254 nm represented by four small inserted figures.

of DCM and MeOH extracts of *M. macrophylla* were tested on ER α - and ER β -dependent activity in HEK293T-ER α and HEK293T-ER β . Both extracts of *M. macrophylla* significantly induced luciferase reporter gene activity in HEK293T-ER α . Figure 2A and 2B show that the DCM extract's activity was lower than that of the MeOH extract. More interestingly, when administered in combination with E2 (10 nM) the DCM extract only significantly antagonized E2 activity at the highest concentration, whereas the MeOH extract showed significant inhibition of the response to E2 at 0.1 and 10 μ g/mL (Fig. 2: A, B). With regard to the HEK293T-ER β cells, the MeOH extract displayed a weak, but statistically significant ER β transactivation at 0.1, and 10 μ g/mL concentrations (Fig. 2D). Surprisingly, the DCM extract not only significantly decreased luciferase reporter gene activity in this system but also inhibited E2-induced activity at all tested concentrations (Fig. 2C).

In order to confirm that the above effects were due to a direct binding of DCM and MeOH secondary metabolites to ERs, we cotreated HEK293T-ER α and HEK293T-ER β with the extracts and ICI. As shown on Supplementary Fig. 2 (available in the online version only), ICI totally abolished the transactivation of ER α and ER β stimulated by both E2 and MeOH. In addition, ICI inhibited the effect of DCM on ER α alone.

Results of 3-day treatment with *M. macrophylla*

Effects on the uterine wet weight and total protein levels: Uterine wet weights of experimental animals after 3 day treatment are shown in Fig. 3A. In response to MeOH and DCM extracts treatments, only the animals that received the DCM extract (300 mg/kg BW) showed an increase in their uterine wet weight by 2-fold (Fig. 3A). In combination with E2, none of the tested extracts exhibited a significant anti-estrogen-like effect (Fig. 3A).

In addition, both extracts increased uterine protein levels. None of them were able to counteract E2V-induced increase of uterine protein levels (Fig. 3C).

Effects on the uterine epithelium

Uterine epithelial heights assessed from 5- μ m sections of paraffin-embedded tissues following hematoxylin-eosin staining are shown in Fig. 3B. Both the DCM and the MeOH extracts of *M. macrophylla* dose-dependently increased uterine epithelial height ($P < 0.05$ for the DCM extract at the doses of 200 and 300 mg/kg as well as for the MeOH extract at the doses of 100 and 200 mg/kg; $P < 0.01$ was obtained only for the MeOH extract at the dose of 300 mg/kg). The response was similar to that of E2V at the dose of 300 mg/kg BW. Both extracts were not able to block E2V-stimulated uterine epithelial growth; however, we noticed an additive effect when the extracts and E2V mixture were given.

Fig. 2. Effects of DCM and MeOH extracts on the activation of estrogen α and β receptors in HEK293T cells in the absence or in the presence of E2 at 10 nM. The effect of DCM and MeOH extracts on estrogen α and β receptors activity in the transiently transfected HEK293T-ER α and HEK293T-ER β cells was investigated by measuring reporter gene-coupled luciferase activity. The relative luciferase units (RLU) were measured in the presence of DMSO (0.1%), E2 (10 nM), DCM, and MeOH extracts. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the control; † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$, compared with E2V.

Effects on the vaginal epithelium

The graphic representation of the vaginal epithelial height (Fig. 4B) shows that E2V induced a 4-fold increase of vaginal epithelial height. Both extracts increased vaginal epithelial height at the tested doses (Fig. 4B). Co-treatment with E2V did not affect the effect of E2V on vaginal epithelium.

Effects on mammary gland

Observations of the 5- μ m sections of paraffin-embedded mammary gland tissues stained with hematoxylin-eosin showed that E2V and the methanol extract of *M. macrophylla* at all tested doses increased the diameter and the lumen of alveoli. An abundant eosinophil secretion in lumen of alveoli was also noted following treatment with either E2V or the MeOH extract. Only the dose of 300 mg/kg BW/d of the DCM extract induced an increment of the diameter and the lumen of alveoli (Fig. 5).

Results of 42-day treatment with *M. macrophylla*

Body weight: The weight of the animals increased from day 0 to day 70, and seemed to be stable thereafter. As expected, the loss of endogenous estrogens due to

ovariectomy resulted in a gradual and significant increase ($P < 0.01$) in body weight in the OVX group compared to the sham group (Fig. 6A). After 42 days of treatment, E2V significantly decreased body weight as compared to the negative control. Only the greatest dose (300 mg/kg BW) of the MeOH extract of *M. macrophylla* could reverse significantly ($P < 0.01$) weight gain initiated by ovariectomy (Fig. 6B).

Uterotrophic effects and vaginal epithelial height

In response to sub-chronic treatment with *M. macrophylla*, the MeOH extract at all tested doses induced statistically significant ($P < 0.01$) increases of uterine wet weight (A), total protein levels (B), uterine (C), and vaginal (D) epithelial height (Fig. 7). The response was however less pronounced than that of E2V. No such effects were noted following administration of the DCM extract of *M. macrophylla* in this treatment regimen.

Fasting serum lipids

The OVX group showed a slight increase of fasting serum total cholesterol levels (Fig. 8A) and fasting serum triglycerides levels (Fig. 8B) compared to sham-operated

Fig. 3. Effects of 3-day treatment with *Millettia macrophylla* extracts combined or not to E2V on the uterine wet weight (A), uterine epithelial height (B), and uterine total protein levels (C). Control = OVX animals treated with the vehicle, E2V = OVX animals treated with estradiol valerate at 1 mg/kg BW, DCM = OVX animals treated with the methylene chloride extract of *Millettia macrophylla*, MeOH = OVX animals treated with the methanol extract of *Millettia macrophylla*. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the control; † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$, compared with E2V.

animals, whereas the HDL-cholesterol levels tended to decrease (Fig. 8C). This has also led to the increase of the atherogenic risk calculated as the ratio of total cholesterol on HDL-cholesterol, due to the loss of ovarian estrogens. E2V (1 mg/kg BW) and the methanol extract of *M. macrophylla* at the dose of 100 mg/kg BW have significantly decreased fasting serum triglycerides and total cholesterol levels after 42 days of treatment. On the other hand, fasting serum HDL-cholesterol levels of rats treated with the plant extracts were not altered compared to the control, while E2V significantly in-

creased it. Similarly, the atherogenic risk (Fig. 8D) was significantly decreased by E2V (1 mg/kg BW) and the methanol extract of *M. macrophylla* at the dose of 100 mg/kg BW.

Discussion

Menopause, the permanent cessation of menses, which marks the end of a woman's reproductive years, is clinically seen as several physical and mental conditions including vasomotor symptoms, histological changes to

Fig. 4. Effects of 3-day treatment with *Millettia macrophylla* extracts combined or not with E2V on the vaginal epithelium: microphotographs (A) and epithelial height (B). Control = OVX animals treated with the vehicle, E2V = OVX animals treated with estradiol valerate at 1 mg/kg BW, DCM = OVX animals treated with the methylene chloride extract of *Millettia macrophylla*, MeOH = OVX animals treated with the methanol extract of *Millettia macrophylla*. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the control. Lu = lumen, Co = stratum corneum, Gr = stratum granulosum, Ge = stratum germinativum, St = Stroma.

Fig. 5. Mammatrophic effects after 3 days of treatment. Control = OVX animals treated with the vehicle, E2V = OVX animals treated with estradiol valerate, DCM = OVX animals treated with the methylene chloride extract of *Millettia macrophylla*, MeOH = OVX animals treated with the methanol extract of *Millettia macrophylla*. Lu = lumen of alveoli, Ep = alveoli epithelium, At = adipose tissue, Se = eosinophil secretion.

Fig. 6. Body weight profile during 126 days of estrogen deprivation (A) and following a sub-chronic treatment (42 days) (B). Sham = Sham operated rats. OVX = ovariectomized animals treated with the vehicle, E2V = OVX animals treated with estradiol valerate, DCM = OVX animals treated with methylene chloride extract of *Millettia macrophylla*, MeOH = OVX animals treated with methanol extract of *Millettia macrophylla*. [#] $P < 0.05$, ^{##} $P < 0.01$, compared with Sham; ^{**} $P < 0.01$, compared with OVX; [†] $P < 0.05$, compared with E2V.

Fig. 7. Graphic representation of uterine wet weight (A), total protein levels in uteri (B), uterine epithelial height (C) and vaginal epithelial height (D) after sub-chronic treatment (42 days). Sham = Sham operated rat. OVX = ovariectomized animals treated with the vehicle, E2V = OVX animals treated with estradiol valerate, DCM = OVX animals treated with the methylene chloride extract of *Millettia macrophylla*, MeOH = OVX animals treated with the methanol extract of *Millettia macrophylla*. [#] $P < 0.05$, ^{##} $P < 0.01$, compared with Sham; ^{**} $P < 0.01$, ^{***} $P < 0.001$, compared with OVX; [†] $P < 0.05$, ^{††} $P < 0.01$, compared with E2V.

estrogen sensitive tissues, dyspareunia, vaginal atrophy, increased risk of developing osteoporosis, insomnia, and cardiovascular diseases. Estrogen is effective for the prevention and treatment of menopausal symptoms. In spite of the efficacy of HRT, the potential side-effects are gaining more and more attention, and the need for developing safer hormone replacement drugs has become more and more urgent. Cameroonian traditional medicine is used because of the richness of its flora that makes

their outstanding herbal formulas more available and at low cost (8, 9, 24). Although some of their therapeutic claims have been documented scientifically with mode and/or molecular mechanism of actions (24 – 28), most like *Millettia macrophylla*, the subject of this study, are still unknown. *M. macrophylla* is used to relieve some menopausal symptoms. *M. macrophylla* is rich in flavonoids and isoflavonoids as shown on the HPLC profiles of DCM and MeOH extracts in our study. Isoflavones in

Fig. 8. Graphic representation of fasting serum total cholesterol (A), triglycerides (B), HDL-cholesterol (C), and total cholesterol on HDL-cholesterol ratio (D) after a sub-chronic treatment (42 days). Sham = Sham operated rats, OVX = ovariectomized animals treated with the vehicle, E2V = OVX animals treated with estradiol valerate, DCM = OVX animals treated with methylene chloride extract of *Millettia macrophylla*, MeOH = OVX animals treated with methanol extract of *Millettia macrophylla*. # $P < 0.05$, compared with Sham; * $P < 0.05$, ** $P < 0.01$, compared with OVX.

particular are known as the main component in soybeans used for the treatment of menopause. Isoflavones structurally fit the binding domain of the estrogen receptor and thereby exhibit properties similar to endogenous estrogens (29). Our present results confirmed that DCM and MeOH extracts have the binding capacity of ER α and ER β . With regard to ER α , DCM and MeOH extracts exhibited estrogenic agonist activity in the absence of oestradiol and showed estrogenic antagonist activity in the presence of oestradiol. In contrast, DCM inhibited the transactivation of ER β in the presence or absence of E2, while MeOH maintained its agonist effect as stated above. This result suggested that both extracts are composed of compounds exhibiting mixed agonistic/antagonistic properties. Flavonoids and isoflavonoids chemical groups and especially isoflavonoids are well known phytoestrogens with mixed effects (14, 30).

In order to further understand the mode of action of *M. macrophylla*, we used an ovariectomized rat model treated for 3 or 42 days after 14 days of hormonal decline as widely described (31). Ovariectomy results in a drastic decrease in uterine weight and vagina thickness at the beginning of the treatment (18, 32), and these changes worsen during the period of subchronical treatment allowing dyslipidemia and decrease in bone mineral density and biomechanical strength as seen in postmenopausal women that we named the postmenopausal-like

model. The model has been used previously by different laboratories to investigate the effects of natural and synthetic products on menopausal symptoms (15, 33, 34). These changes are mostly due to estrogen deficiency. Estrogen can prevent or reverse uterine and vaginal atrophies and dyslipidemia arising from ovarian hormone deficiency by binding to and activating estrogen receptors which in turn modulate the expression of many genes (35). The present data clearly showed that OVX decreased the uterine weight, epithelial height, and total proteins. Administration of MeOH for 3 or 42 days reversed these parameters in ovariectomized rats but not totally while compared to the Sham control and the E2-treated group. Regarding the size of the endometrium, several studies have shown that under the influence of estrogen and estrogenic compounds (genistein, daidzein, alpinunisoflavone), endometrial cells proliferate and differentiate (15, 32, 36–38), which is in close agreement with the results obtained in this work. These results suggest that MeOH extract may have the properties of an estrogen agonist, confirming the in vitro data on ER transactivation. Moreover the lesser uterotrophic effects of MeOH extract after a long treatment period (42 days) may be an indication of its safety in the prevention of uterine tumor. Recently, some authors reported that systemic estrogen replacement restores functional and molecular characteristics of the vaginal muscularis of

ovariectomized rats, morphologically shown by the thickness of vagina and the cornification (39). These data corroborate with ours but with the difference that we did not focus on the contractility of the vaginal smooth muscle.

The results obtained on the mammary gland are striking due to the side effect of HRT and the danger it poses to breast cancer. The histological analyses of mammary glands of the animals treated with the MeOH extract of *M. macrophylla* have shown like E2V, good acini differentiation: well developed ductal epithelia and increment of the number of acini as compared to the control, even though E2V was more potent than the MeOH extract. Several studies suggest that phytoestrogens such as genistein and daidzein reduce the risk of breast and prostate cancer. The preferential binding to ER β of isoflavonoids explains why these compounds reduce the risk of cancer in these organs. The above effect of *M. macrophylla* on mammary gland may be due to the ability of its components to activate ERs as it did in vitro. These results are in accordance with the observations made by Santell and colleagues (40) in which isoflavonoids administration efficiently reversed mammary gland regression induced by ovariectomy.

In the present study, our results showed that ovariectomized animals' body weight increased more rapidly than that of the sham group. This increase in OVX animals' body weight is in accordance with the previous observations reported in the literature (15, 37). Weight gain is the result of complex multifaceted processes, involving particularly the resistance to leptin, a decreased expression of adiponectin and excessive conversion of glucose into fat (41). It could be explained by deposits of fat subsequent to the alteration of the energetic metabolism of lipids and increased abdominal adipose tissue caused by estrogen deficiency engendered by ovariectomy (42). E2V inhibited weight increment induced by ovariectomy. This result is in accordance with the observations of Naaz et al. (43) who reported that estrogen reverses ovariectomy-induced body weight gain. The mechanism of action of estrogen and estrogenic substances in food intake is not yet well identified. However, studies on ERKO mouse suggested that the ER α may be involved in the control of food intake and energy balance modulation (44). However, ER β was characterized as having an orexigenic effect, and it would also be involved in the anorectic effect of ER α (43). MeOH extract of *M. macrophylla* also controlled body weight at the dose of 300 mg/kg. Although we did not assess food intake in this study, it may well be that the secondary metabolites present in *M. macrophylla* have one of the modes of action described above, given that the extracts of *M. macrophylla* could initiate the transactivation of both

ER subtypes in vitro.

OVX rats also developed dyslipidemia related to obesity similar to those seen in women with estrogen deficiency whether by surgery or naturally (15, 45). MeOH could decrease atherogenic risk by alterations of lipoprotein/lipid profiles (Fig. 8). This beneficial effect on lipid metabolism may be due to its ability to activate ERs and induce the transcription of genes involved in lipid metabolism. Therefore *M. macrophylla* may be used like *Pueraria mirifica* (PM), a Thai herb rich in phytoestrogens with a beneficial effect on lipid metabolism in postmenopausal women, which may result from the activation of gene transcription through selective binding of phytoestrogens to ER α and ER β (46). However it should be noted that this beneficial effect of MeOH extract on lipid profile was observed only with the lowest dose (100 mg/kg) and this effect was not dose-dependent. Indeed we noticed that the dose of 100 mg/kg was more effective than 300 mg/kg on the lipid profile. This hormesis type of function, common to many phytoestrogens, has already been observed in many recent studies. Although we do not have insight into the exact cellular and molecular mechanisms underlying this type of function, we suggest that it might be due to saturation of receptors by bioactive compounds present in the extract, which as such lead to the phenomena of down regulation of receptor induced by the higher dose of extract.

The anti-estrogenic activity of the DCM extract observed in HEK293T-ER β could not be reproduced in any of the organs analyzed in the animal experiments (uterus, vagina). This suggests a tissue specific anti-estrogenic activity, i.e., a specific set of co-regulators of estrogen action are needed in a cell/tissue type for the exhibition of the anti-estrogenic activity of the extract of *M. macrophylla*. This result needs a follow-up, as it is the first time that this kind of observation was made in our studies.

In conclusion, we have shown that treatment with the extracts of *M. macrophylla* would produce cardiovascular benefit, as MeOH extract controlled body weight increment induced by ovariectomy, it significantly decreased total cholesterol and triglycerides serum levels, leading to decrease atherogenic risk. It significantly increased the uterine weights and epithelial heights, had mammotrophic effects, it altered ERs activity in vitro, and the effects of both, DCM and MeOH extracts, could be inhibited in vitro by the pure ER antagonist ICI 182,780, suggesting that it may contain compounds that bind to ERs or alter the recruitment of particular co-regulators of ERs. The DCM extract in contrast showed a bifunctional pattern. In vitro this extract showed an estrogenic activity as well as anti-

estrogenic activity depending on the type of estrogen receptors involved. The significance of this later results remains to be elucidated. The estrogenicity and/or anti-estrogenicity of both extracts may be due to the presence of isoflavonoids that are well known phytoestrogens. Taken altogether, these results provide scientific evidence to support the folk use of *M. macropylla* as traditional medicine to alleviate menopausal symptoms among others by village communities in Cameroon. We therefore advise in depth phytochemical studies to further characterize this medicinal plant for its broad usage as an alternative medicine.

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