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Effects of the extracts of some tropical medicinal plants on estrogen inducible yeast and Ishikawa screens, and on ovariectomized Wistar rats

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A total of 33 extracts from 18 Cameroonians plants were studied in two *in vitro* test systems to determine potential estrogenic activities. The estrogenic activities of the extracts, which have shown promising activity on both *in vitro* screens were further investigated *in vivo* on ovariectomized Wistar rats. All 33 extracts were screened in the yeast test-system. Five of these extracts, namely the ethyl acetate extract of the stem bark of *Millettia conraui*, the ethyl acetate extract of the stem bark of *Millettia drastica*, the methanol extract of the leaves of *Bridelia ferruginea*, the methanol extract of the roots of *Pseudarthria hookeri* and the methanol extract of the roots of *Nauclea latifolia* showed interesting estrogenic properties, and were therefore further investigated on alkaline Phosphase induction in Ishikawa cells. The extracts of *Millettia conraui*, *Millettia drastica*, *Pseudarthria hoockeri* and *Nauclea latifolia* showed significant stimulatory effects at 10 and 100 mg/ml doses. The extract of *Bridelia ferruginea* was not further evaluated because of its toxicity on Ishikawa cells. This stimulatory effect was completely inhibited by a combined treatment with the pure antiestrogen ICI (Faslodex^{TM}, 5×10^{-7} M). *In vivo* experiments showed that *per os* administration of 200 mg/kg bw of the extracts of *Millettia conraui* and *Bridelia ferruginea* significantly increased uterine epithelial height by 17.93% and 28.08% respectively compared with uteri of ovariectomized controls after 7 days of treatment. Uterine epithelial height of animals treated with 100 μg/kg bw/d of ethinylestradiol increased by 242.3% in the same experiment. Extracts of *Nauclea latifolia* and *Millettia drastica* had no effect on the uterine epithelial height of ovariectomised rats. 200 mg/kg bw/d of the extracts of *Nauclea latifolia*, *Millettia drastica*, *Bridelia ferruginea* and *Millettia conraui* given orally significantly increased vaginal epithelial height by 15.64%, 24.06%, 51.02% and 58.12% following the same treatment regiment compared to untreated controls. In line with these data was the finding that vaginal epithelial height and vaginal cornification in the presence of each of these extracts was more advanced than in ovariectomized controls although not as prominent as in response to ethinylestradiol treatment. These results suggest that some constituents of the extracts of *Millettia conraui*, *Millettia drastica*, *Pseudarthria hookeri*, *Nauclea latifolia* and *Bridelia ferruginea* may have estrogenic activity.

1. Introduction

It is well known that some plants and common laboratory animal chow have hormonal activity, which sometimes gives rise to significant endocrine effects in grazing or experimental animals. These effects could not be linked to the presence of animal steroid hormones in plants but to naturally occurring non-steroid plant constituents, i.e. phytoestrogens, which elicit estrogen-like effects in one or more target tissues in animals (Mazur and Adlercreutz 2000). Epidemiological data suggest that the consumption of some of these environmental estrogens may be beneficial, for example, by offering protection against breast and prostate cancer, whereas the others may act as endocrine disrupters which as a consequence could affect the endocrine system and may cause developmental and reproductive disturbances (Setchell et al. 1987; Mclachlan et al. 1987; Adlercreutz et al. 1992; Markiewicz et al. 1993). For these reasons, there is the urgent need to characterize the estrogenic properties of plants that are used to cure health related problems. In this report 33 extracts from 18 plants of tropical and subtropical areas were assessed toward potential estrogenic effects. Specific parts of these plants are used in Cameroon as medicinal plants for the treatment of various diseases including female ailments. Experimentally, the estrogenic effects of these extracts were screened

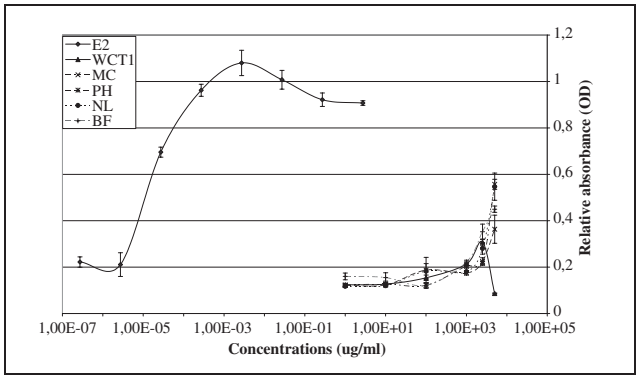

Fig. 1: Estrogenic effect of test substances on the β-galactosidase induction in human estrogen receptor (hER) recombinant yeast. The recombinant yeasts were incubated at 32 °C for 48 h with test compounds (10^{-3}, 10^{-2}, 10^{-1}, 1, 2.5 and 5 mg/mL, which correspond to 1, 10, 10^2, 10^3, 2.5×10^3 and 5×10^3 μg/mL respectively). 17β-Estradiol (10^{-10}–5×10^{-8} M) was used as positive control. Data represent the mean ± SEM (Standard Error of the Mean) of four independent experiments

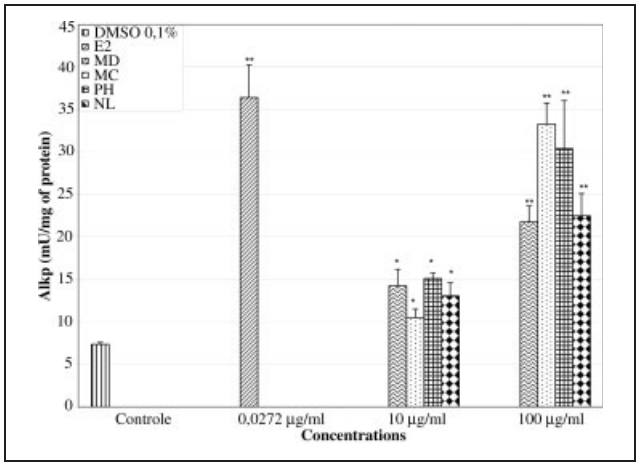

Fig. 2: Dose-response of phytochemicals on the alkaline phosphatase activity in Ishikawa cells. The cells were incubated for 72 h at 37 °C in the presence of test substances. Each treatment was performed in duplicate. The control represents cells treated only with DMSO. Data represent the mean ± SEM of four independent experiments. * $p < 0.05$, ** $p < 0.01$ significantly different from the control group (Analysis of Variance (ANOVA) followed by Dunnett's t-test, a post hoc multiple comparison)

in a yeast estrogen receptor assay; the activity of positive samples was further verified using the Ishikawa cell test-system. The extracts with promising activity in both *in vitro* screens were further tested for estrogenic properties on ovariectomized female Wistar rats thereby mimicking human exposure scenarios.

2. Investigations, results and discussion

2.1. Yeast assay

The estrogenicity of all the 33 extracts was assessed on the estrogen inducible yeast screen ER assay (Fig. 1). The ethyl acetate extract of the stem bark of *Millettia conraui*, the methanol extract of the leaves of *Bridelia ferruginea*, the methanol extract of the roots of *Pseudarthria hookeri* and the methanol extract of the roots of *Nauclea latifolia* showed a dose dependent stimulation of the reporter gene activity, whereas the ethyl acetate extract of the stem bark of *Millettia drastica* showed a different response pattern. Although these extracts exhibited an activity several fold less potent than estradiol, the results were indicative of active constituents in these extracts.

2.2. Alkaline phosphatase assay

The extracts, which showed estrogenic activity on the inducible yeast screen assay, were further tested on Ishikawa cells, except the extract of *Bridelia ferruginea* due to its toxicity on these cells. The results are summarized in Fig. 2. As previously shown 10−7 M E_2, used as positive control, induces maximal stimulation of alkaline phosphatase (Wober et al. 2003). The extracts of *Millettia conraui*, *Millettia drastica*, *Pseudarthria hookeri* and *Nauclea latifolia* showed significant stimulatory effects at all doses tested (10 and 100 μg/mL, which correspond to 0.01 and 0.1 mg/mL). This stimulatory effect was inhibited completely by 5×10^{-7} M ICI 182,780 (Faslodex™), a strong antagonist of the ER, suggesting that the activity observed is ER mediated.

2.3. Uterine weight

The results showed that per os administration of 200 mg/kg bw of the extract of *Millettia conraui*, of the extract of *Bridelia ferruginea* and of the extract of *Nauclea latifolia* have no effect on the uterine weight of ovariectomised rats (Fig. 3), whereas 200 mg/kg bw of the extract of *Millettia drastica* produced a trend towards an increase but without statistical significance (Fig. 3). In the ethinylestradiol-treated group (100 μg/kg bw/d) uterine weight increased seven fold compared to vehicle control. It has been found in another work that regarding the weak uterotrophic effect, the phytoestrogen genistein induced a statistically significant increase in uterine weight only at high doses (Diel et al. 2004), suggesting that this may be the activity pattern of much phytoestrogens.

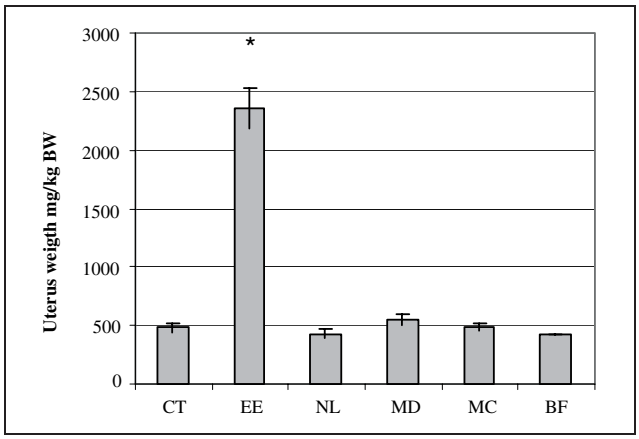

Fig. 3: Graphic representation of uterine weight after 7 days of treatment. CT: control; EE: rats treated with Ethinyl oestradiol; NL, MD, MC, BF: rats treated with *Nauclea latifolia*, *Millettia drastica, Millettia conraui* and *Bridelia ferruginea* respectively. * $p < 0.05$ (Non parametric Mann-Witney U-test)

2.4. Uterine epithelial height

The extract of *Millettia conraui* (200 mg/kg bw) and of the extract of *Bridelia ferruginea* significantly increased uterine epithelial height by 17.93% (from 5.91 ± 0.19 μm to 6.97 ± 0.06 μm) and 28.08% (from 5.91 ± 0.19 μm to 7.57 ± 0.46 μm) respectively compared with uteri of ovariectomized controls after 7 days of treatment (Fig. 4). Uterine epithelial height of animals treated with 100 μg/kg bw/d of ethinylestradiol increased by 242.30% when treat-

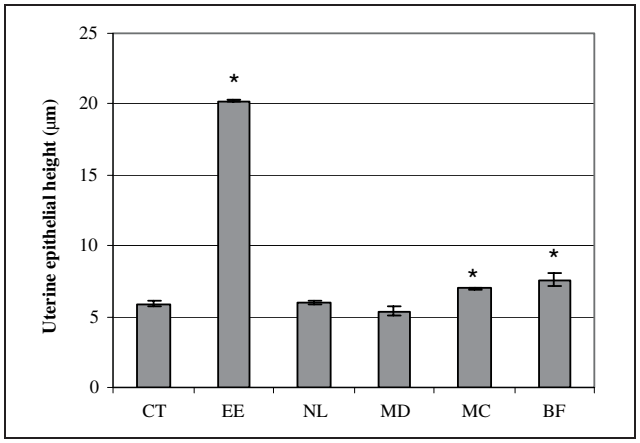

Fig. 4: Graphic representation of uterine epithelial heights after 7 days of treatment. CT: control; EE: rats treated with Ethinyl oestradiol; NL, MD, MC, BF: rats treated with *Nauclea latifolia*, *Millettia drastica*, *Millettia conraui* and *Bridelia ferruginea* respectively. * $p < 0.05$ (Non parametric Mann Witney U-test)

ed 7 days. The extracts of *Nauclea latifolia* and *Millettia drastica* have no effect on the uterine epithelial height of ovariectomized rats (Fig. 4). It is well known that ovariectomy in rats is usually followed by a reduction in cell proliferation and an increase in cell apoptosis. Substituted estrogens or compounds with oestrogen-like activity induce epithelial proliferation and later on an increase in uterine weight (Padilla-Banks et al. 2001). The uterotrophic effect of estrogens has been described as biphasic. Water infiltration into epithelial cells is the earlier marker of oestrogen action in uterus. This uterine water imbibition, due to enhaced microvascular permeability, increases the uterine weight without necessary followed by cells proliferation (Hewitt et al. 2003). The results suggest that the extracts of *Millettia conraui* and *Bridelia ferruginea* have a weak estrogenic effect.

2.5. Vaginal epithelial height

The extracts (200 mg/kg bw/d) of *Nauclea latifolia*, *Millettia drastica, Bridelia ferruginea*, and a *Millettia conraui* given orally significantly increased vaginal epithelial height by 15.64%, 24.06%, 51.02% and 58.12% after 7 days of treatment respectively compared to untreated ovariectomised rats (Fig. 5). Vaginal epithelial height of animals

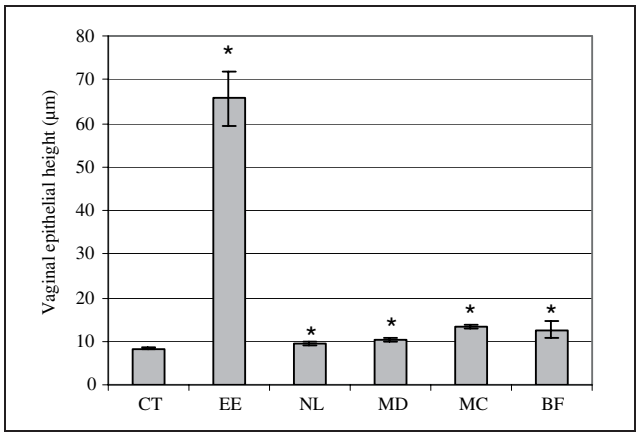

Fig. 5: Graphic representation of vaginal epithelial heights after 7 days of treatment. CT: control; EE: rats treated with Ethinyl estradiol; NL, MD, MC, BF: rats treated with *Nauclea latifolia*, *Millettia drastica*, *Millettia conraui* and *Bridelia ferruginea* respectively. * $p < 0.05$ (Non parametric Mann-Witney U-test)

treated with 100 µg/kg bw/d of ethinylestradiol increased by 690% after 7 days of treatment when compared with untreated controls. In line with these data was the finding that vaginal epithelial height and vaginal cornification in the presence of each of these extracts was more advanced than in ovariectomized animals although not as prominent as in response to ethinylestradiol treatment. Measurement of the vaginal epithelial height has early been shown to be a more sensitive parameter (Diel et al. 2001). Increase in the uterine weight and epithelial cell proliferation are estrogenic effects mediated by ERα (Cooke et al. 1997; Padilla-Banks et al. 2001; Jefferson et al. 2002). The estrogenic activity of *Millettia drastica* and *Millettia conraui* may be due to the presence of flavonoids and isoflavonoids in Millettia genus (Yankep et al. 2001), as the phytoestrogen yet described include isoflavonoids, lignans and also some of the flavones, flavanones, chalcones, coumestans, stilbenes (Wiseman 1999).

Of the plants that have been found to exhibit promising estrogenicity in this study, three are members of the Leguminosea family, which is reputed to yield isoflavonoids (Dewick 1994; Yankep et al. 2001), which are known for their estrogenic properties (Mazur and Adlercreutz 2000). We have also shown in our work that isoflavones derived from *Millettia griffoniana* have estrogenic properties (Ketcha et al. 2006). We look forward for a possibility to screen the extracts in a ER-β assay. We also wish to identify the active principles of the promising extracts. This may be achieved through a bioassay guided phytochemical study of the extracts. In addition, once a promising candidate molecule would have been identified, we may perform *in vivo* uterotrophic assays thereby establishing organ specific gene expression finger prints of the estrogenic activity of the extracts and isolated substances. In this way potential selective estrogen receptor modulating (SERM) activities of the candidate molecules may be identified.

3. Experimental

3.1. Substances

17β-Estradiol obtained from Sigma (Deissenhofen, Germany), and ethinylestradiol from Laboratoires EFFIK (France) served as the reference substances for *in vitro* and *in vivo* experiments respectively. Plant extracts and 17β-estradiol were dissolved in DMSO for *in vitro* assays, whereas ethinylestradiol and the extracts, were dissolved in ethanol/Tween80/distilled water (1 : 1 : 8) for *in vivo* experiments on Wistar rats.

The plant extracts screened in this study were obtained from two different sources. Firstly those plant extracts which were known for their traditional uses as remedies for female ailments and were also described in pharmacopeias as medicinal plants were included. They were previously extracted and used for various applications in the Phytochemistry laboratory of the Faculty of Science, University of Yaounde 1. Plants, plant parts used and extraction procedures are summarized in the upper part of the Table. As second source, an ethnobotanical survey oriented on a search for plants used in different geo-cultural village communities in Cameroon in the traditional treatment of menopausal complaints and female infertility was carried out. Plants obtained from this inquiry were collected, identified with the help of the Cameroon National Herbarium (Yaounde), where voucher specimens are deposited for further references. Plant materials collected were dried, ground (300 to 500 g) and extracted successively in dichloromethane and methanol. For each solvent, extraction was performed at room temperature for 72 h, before evaporation of the solvent under reduced pressure using a rotary evaporator. The plants, plant parts used and the extracts obtained are summarized in the lower part of the Table. In total 33 extracts from 18 species were screened.

3.2. Estrogenicity in cell systems

For the assessment of the estrogenicity, a widely used estrogen inducible yeast screen estrogen receptor assay was used (Routledge and Sumpter 1996). The yeast strain contained both a stably transfected estrogen receptor-α (ER-α) construct and an expression plasmid carrying estrogen-responsive sequence controlling the reporter gene lac-z encoding the enzyme

Table: Overview of the plants and the extraction procedures

Plant species	Family	Plant part used in traditional application	1st extraction solvent	2nd extraction solvent
I. Medicinal plant extracts under phytochemical studies in the department of Organic Chemistry				
Celtis gomphophylla	Cesalpiniaceae	Stem bark	Dichloromethane/methanol (1 : 1)	
Celtis tessmanii	Cesalpiniaceae	Stem bark	Dichloromethane/methanol (1 : 1)	
Millettia conraui	Fabaceae	Stem bark	Ethylacetate	
Millettia drastica	Fabaceae	Root bark	Ethylacetate	
Millettia drastica	Fabaceae	Stem bark	Ethylacetate	
Millettia drastica	Fabaceae	Leaves	Ethylacetate	
Erythrina milbraedii	Fabaceae	Root bark	Ethylacetate	
Erytrina addisoniae	Fabaceae	Stem bark	Dichloromethane	Methanol
II. Species obtained from our ethnobotanical survey				
Bridelia ferruginea	Euphorbiaceae	Stem bark	Dichloromethane	Methanol
Bridelia ferruginea	Euphorbiaceae	Leaves	Dichloromethane	Methanol
Hypodaphnis zenkeri	Lauraceae	Stem bark	Dichloromethane	Methanol
Scorodophloeus zenkeri	Cesalpiniaceae	Stem bark	Dichloromethane	Methanol
Guibourtia tessmanii	Euphorbiaceae	Stem bark	Dichloromethane	Methanol
Monodora mirystica	Annonaceae	Fruits	Dichloromethane	Methanol
Aframomum citratum	Zingiberaceae	Fruits	Dichloromethane	Methanol
Tetrapleura tetraptera	Mimoseae	Fruits	Dichloromethane	Methanol
Piper guineense	Piperaceae	Fruits	Dichloromethane	Methanol
Mondia whitei	Periplocaceae	Roots	Dichloromethane	Methanol
Pentadiplendra brazzeana	Pentadiplendraceae	Root bark	Dichloromethane	Methanol
Pseudarthria hookeri	Fabaceae	Root bark	Methanol	
Nauclea latifolia	Rubiaceae	Root bark	Methanol	

β-galactosidase. Estrogenic activity from the enzymatic hydrolysis of chlorophenol red β-D-galactopyranoside was read at 540 nm using a colorimetric assay. In a concentration dependent analysis of reporter gene activity, half maximal induction of β-galactosidase activity was a direct measure for the activity of the compound on the ER-α. All the 33 extracts were screened with this test system. Further evaluation of the estrogenicity of those extracts which showed interesting activities in the yeast screen, was done in the human endometrial adenocarcinoma cell line Ishikawa. This method is based on induction of alkaline phosphatase by estrogens (Wober et al. 2003). In brief, Ishikawa cells were cultured in DMEM/F12 without phenolred medium treated with 5% dextran-coated charcoal (DCC), FCS (Gibco, Karsruhe) and insulin-transferrin-selenium A (Gibco BRL). Cells were kept in plastic culture flasks at 5% CO_2 and 37 °C and harvested by a brief exposure of trypsin (0.05%) EDTA at 37 °C. After inhibition of the action of trypsin by addition of 5% DCC containing medium and pelleting, the cells were resuspended in culture medium and seeded in 12 well plates at the required density of 125.000 cells per well. The tested substances, diluted in DMSO, were tested in a dose-dependent manner. After 72 h incubation, cells were harvested and resuspended in reaction buffer (274 mM mannitol, 100 mM CAPS, 4 mM $MgCl_2$, pH 10.4). Using ultrasonic desintegration cells were lysed at 4 °C. Alkaline phosphatase activity was assayed by a method involving the hydrolysis of *p*-nitrophenylphosphate to *p*-nitrophenol at pH 10.4 and the spectrometric determination of the kinetics of the product using the BCA-kit (Sigma).

3.3. Animals

Wistar rats are routinely breed in the laboratory of Animal Physiology, University of Yaounde I (Cameroon). They had free access to standard soy free rat diet (Ssniff RM R/M-H, 10 mm, sojafrei + PE-Diet, Ssniff GmbH, Soest, Germany) and water.

3.4. Uterotrophic assay

Female Wistar rats aged 10 weeks and weighing between 180–200 g were obtained from the animal house of the laboratory of Animal Physiology, University of Yaounde I (Cameroon) and were ovariectomised. They had free access to a standard soy free rat chow (Ssniff R10-Diet) purchased from Ssniff GmbH, Soest, Germany; and water. After 14 days of endogenous hormonal decline, the animals were randomly allocated to 7 days treatment per os with: ethinylestradiol (100 μg/kg bw/d) and different extracts (200 mg/kg bw/d). The control group received the vehicle. Animals were sacrificed after light anaesthesia with ether inhalation. Uterine and vaginal epithelial heights were assessed from 5 μm section of paraffin embedded uterine and vaginal tissues. Following azan and hematoxylin-eosin staining, uterine and vaginal epithelial height were assessed on microphotography using a complete Zeiss equipment consisting of a microscope Axioskop 40 linked to a computer where the image is transferred, edited and analysed with the MRGrab1.0 and AxioVision 3.1 software, all provided by Zeiss (Hallbermoos, Germany). Animal housing and all *in vivo* experiments were carried out following the guidelines of the institutional Ethic Committee of the Cameroon Ministry of Scientific Research and Technology Innovation, which has adopted the guidelines established by the European Union on Animal Care (CEE Council 86/609).

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