International Journal of Phytomedicine 8 (2016) 366-378
http://www.arjournals.org/index.php/ijpm/index

Original Research Article

ISSN: 0975-0185

Evaluation of the estrogenic properties of aqueous extracts of *Tragia benthamii* Baker (Euphorbiaceae) and *Graptophyllum pictum* (Acanthaceae) and their ability to alleviate some menopausal symptoms induced by ovariectomy in Wistar rats

Germain Jean Magloire Ketcha Wanda[1]*, Sefirin Djiogue[2], Sosthène Onésiphore Djoussi Njimfo[2], Charline Florence Awounfack[2], Dieudonné Njamen[2].

***Corresponding author:**

Germain Jean Magloire Ketcha Wanda

[1]Department of Psychology, Faculty of Arts, Letters and Social Sciences, the University of Yaoundé I, Cameroon.
[2]Department of Animal Biology and Physiology, Faculty of Science, the University of Yaoundé I, Cameroon.

Abstract

Tragia benthamii Baker (Euphorbiaceae) and *Graptophyllum pictum* Linn (Acanthaceae) are two Cameroonian medicinal plants traditionally used against female reproductive tract disorders, during and after the reproductive period, and as an abortifacient. Since there were no scientific data supporting the above claims and pharmacological studies characterizing their estrogenic properties, we therefore aimed to evaluate their ability to induce estrogen-like effects on primary estrogens targets, uterine, vagina and mammary gland; as well as their ability to alleviate hot flushes in ovariectomized adult rats. For this purpose, we applied a 3-day uterotrophic assay to determine the estrogenic effects of each extract and the mixture of both plants as used by traditional practitioners. The extracts were administered orally for 3 days to the 10 to 12 weeks aged ovariectomized rats. The results obtained showed that the aqueous extract of *T. benthamii* at the dose of 500 mg / kg BW, the aqueous extract of *G. pictum* at all the tested doses as well as the aqueous extract of the mixture at 275 mg / kg body weight induced a significant increase ($p < 0.01$) of the uterine epithelium thickness. In addition, the aqueous extract of *T. benthamii* at the dose of 500 mg / kg BW, as well as the aqueous extract of the mixture at the doses of 50 and 275 mg / kg induced acinar development and eosinophil secretions. These results are proof of estrogen-like effects of *T. benthamii* and *G. pictum* and therefore justify the traditional use of these plants. This suggests the presence in these plants, of secondary metabolites with estrogenic properties, can induce cell proliferation and correct disorders of post-oophorectomy œstrogénopénia in the Wistar rats and therefore menopausal disorders.

Keywords : *Tragia benthamii, Graptophyllum pictum*, uterotrophic effect, phytoestrogens, hot flushes

Introduction

Estrogens, especially 17β-estradiol, are pleiotropic gonadal steroids that affect many physiological functions including reproduction, bone metabolism, the cardiovascular system and brain function [1]. The importance of estrogens is even more critical during the menopausal period. This period that marks the end of the reproductive life span of women is characterized by the cessation of estrogen production. This brutal drop in circulating estrogen results in the development of associated pathophysiological conditions such as vasomotor instability (hot flushes), insomnia, loss of libido, depression and vagina dryness [2], genito-urinary atrophy, osteoporosis. These troubles may be so severe that they require a therapy [3].

Vasomotor instabilities for instance are the hallmark of estrogen deficiency at menopause, and greatly affect women's quality of life. Over the decade, Hormone replacement therapy (HRT) has been successfully used to treat the symptoms of menopause because estrogen has strong suppressive effects on climacteric complaints. Recent studies, however, associated HRT with adverse effects after long-term use, such as increased risks of endometrial and breast cancers, stroke and pulmonary thromboembolism [4, 5, 6]. Moreover, women are reluctant to use HRT because of undesirable side effects such as irregular bleeding and safety, especially risk of breast cancer [7]. Thus many women are increasingly relying on

DOI:10.5138/09750185.1914

natural health remedies as alternative therapies to treat menopausal symptoms [8]. Kaufert et al(1998) [9] have reported that 80% of women aged 45–60 years were using non-prescription therapies to manage menopausal symptoms. More recently, Ferrari (2009) [10] reported that in daily practice conditions, high doses of isoflavones, particularly genistein, can be used for the management of hot flushes in postmenopausal women not treated with HRT due to their early onset of action, efficacy and safety. There are efforts to find new compounds that exert selective effects by acting as an estrogen receptor (ER) antagonist on neoplastic or normal breast and uterine tissues, and as an ER agonist on estrogen-responsive tissues like bones, liver and the central nervous system. In this search, ethnobotany and pharmacognosy are being used as guide to lead scientists toward different sources and classes of compounds, and the tropical flora, by virtue of its diversity, continues to provide new leads.

Tragia benthamii (Baker), a climbing herb of the family Euphorbiaceae, widely spread in west, central and south Africa [11],is traditionally used in Cameroon as an abortifacient, anti-microbial, against generalized pain, urethritis and infertility in women [12]. The stem and leaves of *Graptophyllum pictum*, an herbaceous of the family Acanthaceae, are traditionally used as anti-microbial, against inflammation, ulcers, abscesses, haemorrhoids, anaemia [13]. A number of scientific investigations and some authors have reported its anti-inflammatory properties [14], oxytocin and anti-implant [15], and hypoglyceamic activity [16]. In Cameroon, a mixture of both *T. benthamii* and *G. pictum* is used by traditional healers to treat secondary amenorrhea and generalized infertility. Since no pharmacological studies have been reported characterizing the estrogenic properties of *T. Benthamii* and *G. pictum* extracts and the mixture of both plants, this study therefore aims to provide a scientific base for the traditional use of this mixture of two plants and assess its interest in menopause related disorders.

We have used a postmenopausal-like model of ovariectomized Wistar rats to evaluate the effects of the aqueous extract of the two plants on some menopausal disorders. The biological activity was evaluated on female rat estrogen primary target organs, the uterus and the vagina. We specifically evaluated the ability of our plants preparation to stimulate the proliferation of uterine and vaginal epithelia, as well as their ability to induce eosinophil secretions in the mammary gland, and to reduce the average duration, number and frequency of hot flushes.

Materials and Methods

Chemicals

The17β-estradiol valerate (E2V) (Progynova®) was purchased (obtained) from Delpharm (Lille, France). Penicillin (xtapen®) was manufactured by CLBC Zhongnuopharmaceutical® (Zhejiang, China), Diclofenac (DICLOECNU®) by ECNUpharceutical CO LTD (Yanzhou City,China). ValiumRochewas manufactured by SASCenexi Fontenay-sous-Bois(France),and Ketamine was obtained by Troikaa Pharmaceuticals Ltd (Gujarat, India).

Plant material: Extraction and isolation

The aerial parts of *Tragia benthamii* Baker and *Graptophyllum pictum* Linn were harvested (collected) in Mbalmayo in the central region of Cameroon. A voucher specimen of *T. benthamii* was authenticated (N 586/NHC) at the National Herbarium of Cameroun. For

G. pictum no reference of the plant was found at the Cameroun National Herbarium. It sample was then identified by Dr. TCHIENGUE Bartholomew T., a botanist of the National Herbarium of Cameroon (HNC-IRAD).

The decoction was prepared following the recommendations of the traditional healers.After being harvested, weighed and washed, 235 g of *T. bentamii*, 296g of *G.pictum*as well asthe mixture of two plants (235 g *T. bentamii* + 296 g of *G. pictum*) were respectively boiled in 10 liters of water for 45 minutes. Each solution was filtered with Whatman No. 4 filter paper. The filtrate was frozen and then lyophilized resulting into 5g of *T. bentamii*, 6g of *G. pictum* and 13g for the mixture of two plants dried extract (slightly brown powder), representing respectively 2.13%, 3.4% and 2.45% yield.

Animals

Juvenile female Wistar rats aged 10 to 14 weeks old (average weight 150 g) were obtained from the breeding facility of the laboratory of Animal Physiology, University of Yaoundé 1 (Cameroon).They were provided tap water ad libitum and a soy-free rat diet (SSniff R10-Diet, SSniff GmbH, Soest, Germany). All animals' husbandry handling conditions were in accordance with the guidelines of the institutional Ethic Committee of Cameroon's Ministry of Scientific Research and Technological Innovation, which has equally adopted the guidelines established by the European Union on Animal Care (CEE Council 86/609; Reg.no.FWA-IRD0001954)

Study design

Experiment 1: The 3-day uterotrophic assay with crude extracts

Prior to the treatment, Estradiol valerate and the crude extract of *T. benthamii* and *G. Pictum* were dissolved in distilled water used as vehicle, and we obtained a homogenized solution. The doses to be administered were prepared following the proposal of the traditional healer. In order to obtain a dose response curve, 3 different doses (50, 275 and 500mg/kgBW) were generated.

Fifty five female Wistar rats were bilaterally ovariectomized (OVX) using the dorsal approach [17], under Diazepam and ketamine anesthesia (respectively 10 mg/kg and 50 mg/kg BW ;i.p.). After 14

days of endogenous hormonal decline, animals were randomly distributed into 11 groups of five animals each. The first group or OVX group received vehicle only (distilled water) and the second group received estradiol valerate (E2V) as standard drug at the optimal dose of 1mg/kg BW per day. The third, fourth and fifth groups received, 50 mg / kg BW / day of the aqueous extract of respectively *T. benthamii, G. pictum* and their mixture. The sixth, seventh and eighth lots were given a dose of 275 mg / kg BW / day, respectively the aqueous extract of *T. benthamii*, *G. pictum*and *T. benthamii* + *G pictum*. The ninth, tenth and eleventh lots received at the dose of 500 mg / kg BW / d, the aqueous extract of respectively *T benthamii*, *G pictum* and *T benthamii* + *G pictum.*

All treatments were given by oral route (2 mL/250 g) for 3 days. Twenty four hours after the last administration, animals were weighed and sacrificed by decapitation (after 12 h of fasting-). The Mammary gland, vagina and uterus were collected. The uterus was immediately weighed. The uterine wet weight, uterine and vaginal epithelial thickness and mammary gland were assessed as described before by Zingue et al. [18].

Experiment 2. : Measurement of hot flushes

The physiological rat model for hot flushes, introduced by Berendsen et al.[19] was used to assess the ability of *G. pictum* extract to alleviate hot flushes, with the slight difference that data loggers were used instead of a telemetric transmitter. Data loggers were used to monitor the core temperature changes in the animals at 2 min intervals for 72h, as previously described by Njamen et al.[20] and Nkeh-Chungag et al. [21]. In this study, data loggers were pre-set to start measuring core temperatures 12h before the beginning of the treatment until the end of treatment. Twenty acclimatized rats were bilaterally ovariectomized (OVX) under valium and ketamine anesthesia (respectively 10 and 50 mg/kg BW; i.p.) and at the same time underwent the implantation of data loggers protected in sterilized neutral wax into their abdominal cavities. Only the doses of extracts that showed a promising estrogenic effect on the previous experiment were retained for this experiment.

After 14 days of endogenous hormonal decline, animals were randomly distributed into four groups of five rats each.

The first lot, as negative control, received the vehicle (distilled water). The second group as a positive control received estradiol valerate (E2V) at a dose of 1 mg / kg BW. The last 2 lots were given the aqueous extracts of *G. pictum* at the doses of 50 and 275 mg / kg BW.

They were treated for 3 days and all treatment was given by oral route (2 mL/250g). Twenty-four hours after the last administration, animals were sacrificed by decapitation after 12h of fasting, and the data loggers recovered. Data (central body temperature) was retrieved from loggers and analyzed using the ACR Trend Reader for Smart Button Software. The mean core temperature change (core temperature) was determined as previously described by Maswood et al. [22] and plotted in a 6h intervals. Hot flushes were considered for any internal temperatures 38 C. The total number of hot flushes, the average of these hot flush durations and the frequency of hot flushes were determined as described by Nkeh-Chungag et al. [21]

Histological analysis

Uterine and vaginal epithelial heights and mammary glands differentiation were assessed from 5-µm sections of paraffin-embedded uterine, vaginal and mammary gland tissues. Following the hematoxylin–eosin staining, uterine and vaginal epithelial heights and mammary glands differentiation were assessed on microphotography using the complete Zeiss equipment consisting of a microscope Axioskop 40 linked 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).

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Analysis of the results was made using one-way ANOVA followed by the Dunnett's test for multiple comparisons through Graphpad software version 3.10. All treated groups were compared to the ovx-control. The significance was determined at p 0.05 and p 0.01 with the level of risk of 5% and 1% respectively.

Results

Effects of aqueous extracts of *T. benthamii*, *G. Pictum* and their mixture on some primary targets of estrogen

Effects on uterine weight

The uterine wet weights in ovariectomized (OVX) rats following the administration of different doses of aqueous crude extract of *T. benthamii, G. pictum* (50, 275, and 500 mg/kg BW) and the w/w mixture of both plants extracts (50, 275, and 500 mg/kg BW) are shown in Fig. 1. given to ovariectomized rats, induced a mild trend towards an increase of uterine weight, but this increase did not reach statistical significance, while E2V 1 mg / kg BW increased uterine weight 9.9 fold compared to control.

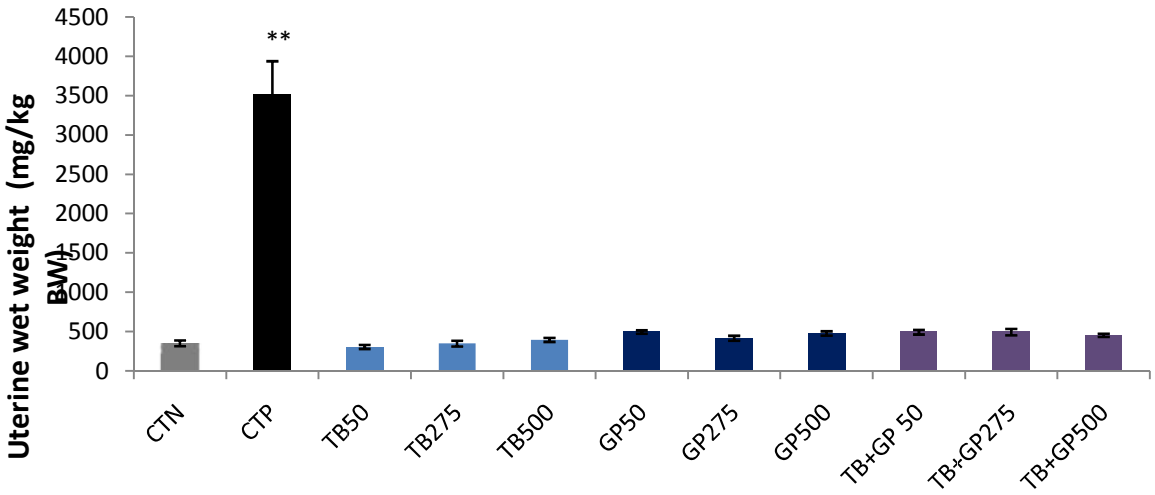


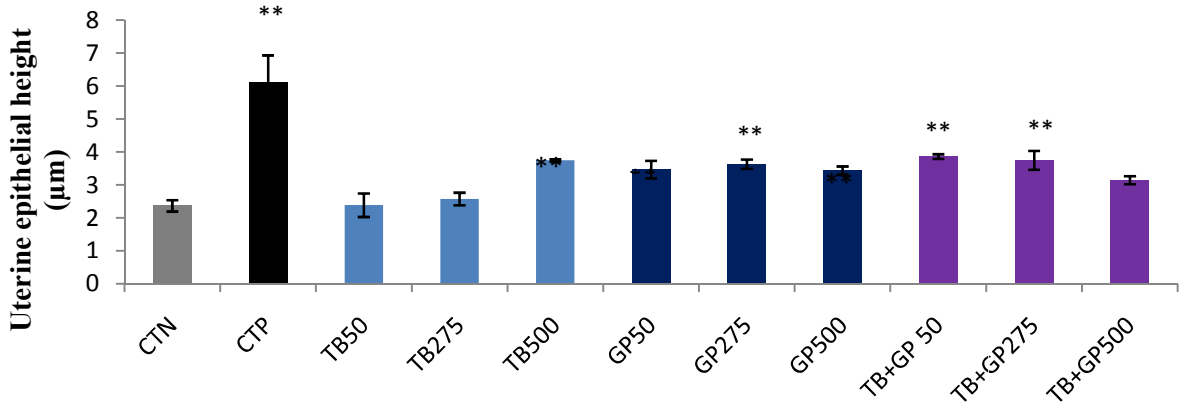
Figure1: Uterine wet weight: uterine wet weight of ovariectomized Wistar rats after daily gavage of vehicle (OVX), Estradiol valerate 1 mg / kg BW (E2V) and three doses (50, 275 and 500 mg / kg BW crude extract of *T. benthamii*, *G. pictum* and the mixture of both plants extracts for 3 days. Each group consisted of 5 animals. Data represent mean ± SEM. ** indicates a significant difference as compared to the vehicle-treated animals. P < 0.01. (ANOVA followed by Dunnett's test).

Effects on the size and histomorphology of the uterine epithelium

The results presented in Figure. 2 (A) show that three days after treatment, the uterine epithelium high was 2.51 ± 0.08 microns in the negative control while E2V induced a significant 168.12% increase (p <0.01) of this paprameter (6.73 ± 0.51 µm). In addition, a significant increase was also observed with the *T. benthamii* aqueous extract at the dose of 500 mg / kg BW (50.59% p <0.01). Furthermore a significant increase was also found with the aqueous extract of *G. pictum* at the doses of 50 (47.01% p <0.01), 275 (41.02% p <0.01), and 500 (40.63%: p <0.01) mg/kg BW. Moreover, the aqueous extract of the mixture of both plants induced a significant increase in the uterine epithelium high at the doses of 50 (52.22% p <0.01) and 275 (58.88% p <0.01) mg/kg PC.

Figure. 2 (B) shows the micrographs of the uterine epithelium of rats after treatment. E2V at the dose of 1 mg/kg BW, showed a 2.5-fold (P < 0.01.) increase of uterine epithelial thickness. Aqeous extract of *T. benthamii* (500 mg/kg BW), and *G. pictum* (275 mg/kg BW) and the mixture of both plant extract (50, 275 and 500 mg/kg/BW) slightly modified the shape of the uterine epithelium. These effects were materialized in histological sections by the formation of a tall cuboidal to columnar epithelium (the cylindrical shape) containing large cells following E2V treatment while in the OVX group uteri consisted of a low cuboidal epithelium

A



B

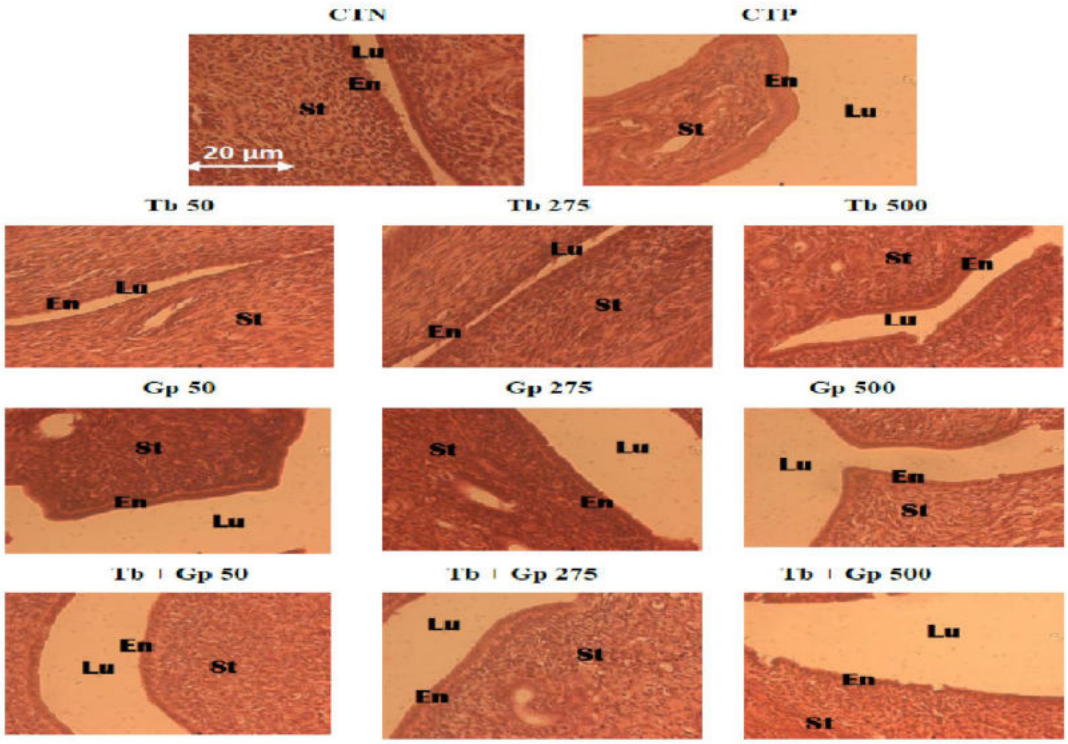


Figure 2: Effects of a 3-day treatment with *T. benthamii*, and *G. pictum* extracts (50, 275, and 500 mg/kg BW) on the uterine epithelial thickness (A) and microphotographs showing the effect of a 3-day treatment on the size of the uterine epithelium of ovariectomized rats(B). OVX = OVX animals treated with the vehicle; E2V = OVX animals treated with estradiol valerate at 1mg/kg BW; AE= OVX animals treated with the Aqueous extract (50, 275, and 500 mg/kg BW) of *T. benthamii*, *G. pictum* extracts and the mixture of both plants (Tb+Gp). Each group consisted of 5 animals. Data represent mean ± SEM. ** indicates a significant difference as compared to the vehicle-treated animals. P < 0.01. (ANOVA followed by Dunnett's test). Lu:uterine lumen;En: Endometrium; St:Stroma.

Regarding the comparison of the effects of aqueous extracts of *T. benthamii*, and *G. pictum* to that of their mixture on the uterine epithelium high, there is a significant difference between the effects of the aqueous extract of *T. benthamii* and that of the mixture at a the dose of 50 mg/kg BW, while between the effects of the aqueous extract of *G. pictum* and that of the mixture, no significant difference was observed at the same dose (Figure. 2A). Moreover, at the dose of 275 mg/kg BW, there is a significant difference between the effect of *T. benthamii* and that of the mixture; As well as the effects of *G. pictum* when compare to the mixture (Figure. 2B).

Effects on the size and histomorphology of the vaginal epithelium

The effects of the various treatments after a 3 days treatment can be observed on the figure 3. E2V at the dose of 1 mg / kg BW significantly increased ($p < 0.01$) the vaginal epithelium high. The thickness increased by 548% compared to the control. No significant increase of the high of the vaginal epithelium was observed with our extracts at all the tested doses after 3 days of treatment.

Regarding vaginal epithelial thickness, the microphotographs of vaginal epithelium of the OVX group (negative control) consisted of a single layer of squamous cells, the germinative layer (stratum germinativum Ge). After treatment with E2V 1 mg/kg BW, the vaginal epithelium was stratified giving place to three cell layers: the germinative layer at the baseline, the granular layer in the middle and the cornea surface releasing layer of the granular layer. No similar stratification of vaginal epithelium was induced by our extracts at all the tested doses.

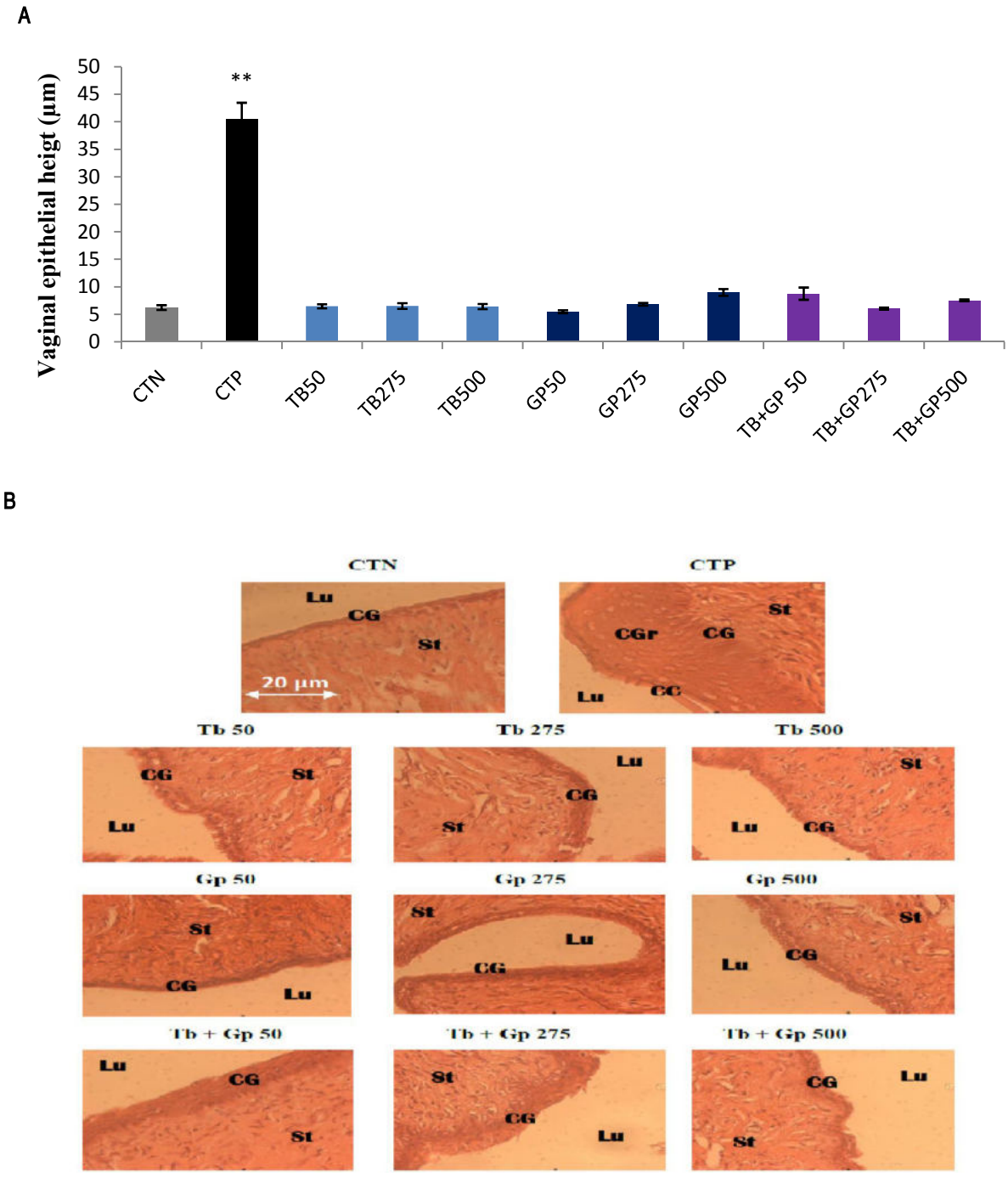


Figure 3: Effects of a 3-day treatment with *T. benthamii*, and *G. pictum* extracts (50, 275, and 500 mg/kg BW) on the vaginal epithelial thickness (A) and microphotographs showing the effect of a3-day treatment on the high of the vaginal epithelium of ovariectomized rats(B). OVX = OVX animals treated with the vehicle; E2V = OVX animals treated with estradiol valerate at 1mg/kg BW; AE= OVX animals treated with the Aqueous extract (50, 275, and 500 mg/kg BW)of *T. Benthamii* (Tb), *G. Pictum* (Gp) extracts and the mixture of both plants (Tb+Gp). Each group consisted of 5 animals. Data represent mean ± SEM. ** indicates a significant difference as compared to the vehicle-treated animals. . P < 0.01. (ANOVA followed by Dunnett's test).Lv=vaginallumen,CC=stratum corneum,CGr=stratum granulosum, CG=stratum germinativum, St=Stroma.

Effects on mammary gland

After 3 days of treatment, at the level of the mammary glands (Figure 4), there is an increase in size and lumen of acini, a clear differentiation of cell layers constituting the acinus, an increase of eosinophils secretions in the lumen of acini and lobules of large size in animals treated with E2V 1 mg/kg BW, the mixture of the aqueous extracts of *T. benthamii*, and *G. pictum*, at the doses of 50 and 275 mg /kg BW and the aqueous extract of *T. benthamii* at the dose of 500 mg/kg BW.

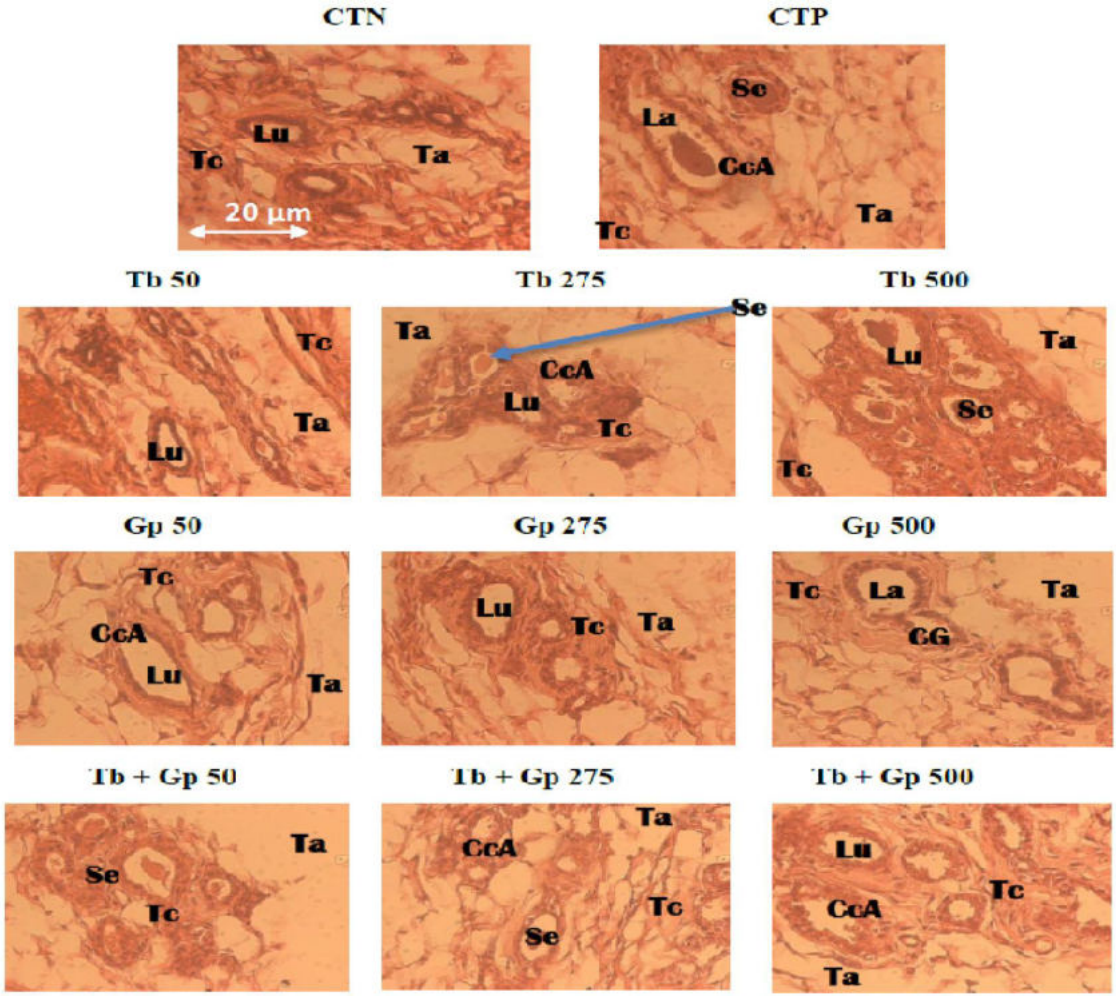


Figure 4: Effects of a 3-day treatment with *T. benthamii*, and *G. pictum* extracts (50, 275, and 500 mg/kg BW) on mammary gland. OVX = OVX animals treated with the vehicle; E2V = OVX animals treated with estradiol valerate at 1mg/kg BW; AE= OVX animals treated with the Aqueous extract (50, 275, and 500 mg/kg BW)of *T. benthamii* (Tb), *G. pictum* (Gp) extracts and the mixture of both plants (Tb+Gp). La= lumen of alveoli or acini; CcA=celllayer of theacini; Se =eosinophilicsecretion;
Ta =Adipose tissue;Tc =Connective (conjunctive) Tissue.

Effectsof *G.pictum* on hot flushes

Effects on the average duration of hot flushes

Fourteen days after the ovariectomy followed by three days of treatment, E2V significantly ($p < 0.01$) reduced the average duration of hot flushes compared to that of the NTC negative control. This duration decreased from 3.59 ± 0.49 hours in the control to 1.22 ± 0.47 hours in the E2V treated group, a decrease of 294.27%.The treatment with the aqueous extract of G. pictum, did not significantly reduce the average duration of hot flushes at the doses tested (50 and 275 mg / kg BW). (Figure. 5)

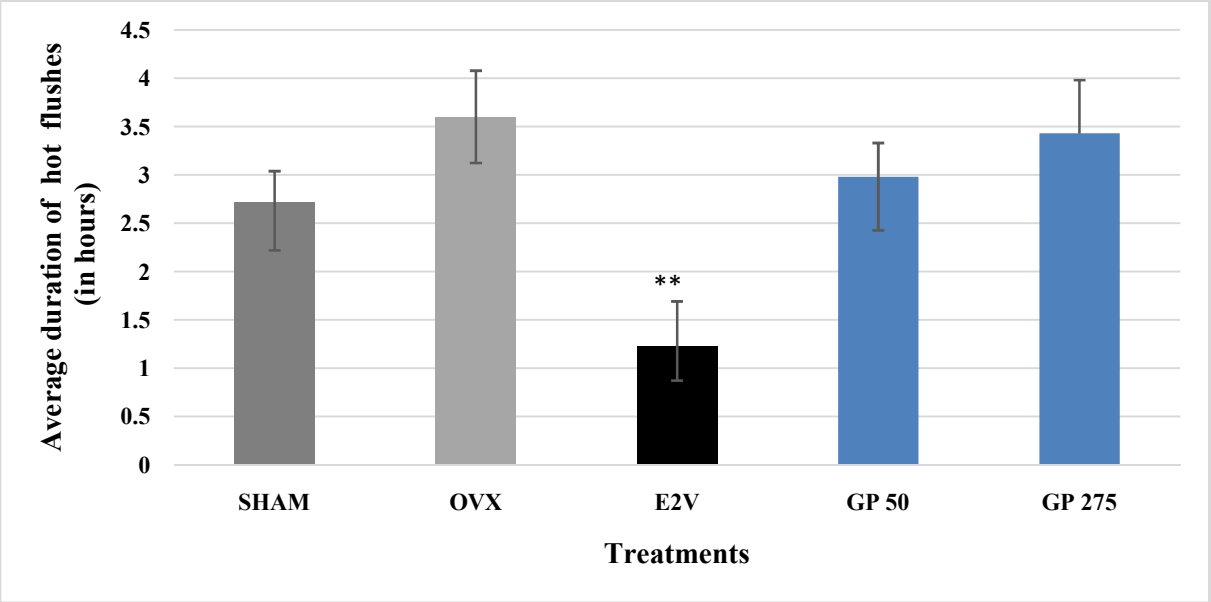


Figure 5: Effects of a 3-day treatment with *G. pictum* extracts on average duration of hot flushes. SHAM=Sham operated rats treated with the vehicle; OVX=OVX animals treated with the vehicle; E2V=OVX animals treated with estradiol valerate at 1mg/kg BW; GP=OVX animals treated with the aqueous extract of *G. pictum.* (50 and 275 mg/kg BW).** P<0.01 as compared to the control.

Effects on the total number of hot flushes

After the treatment, only the E2V significantly (p <0.01) reduced the total number of hot flushes. It decreased 2.5-fold compared to control. The treatment with aqueous extract of *G. pictum*, did not significantly reduce the total number of hot flushes. However, there is a slightly decrease in the total number of hot flushes at a dose of 275 mg / kg BW, compared control (Figure. 6).

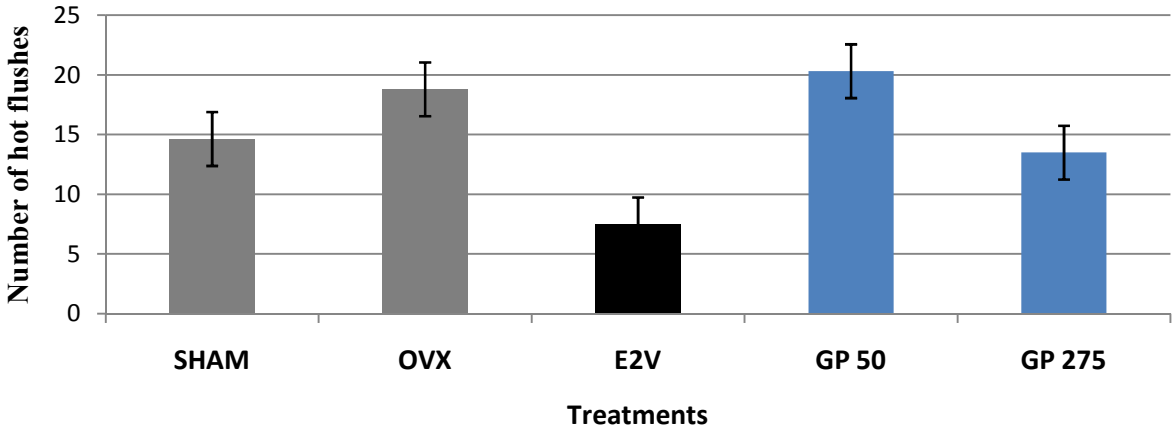


Figure6: Effects of a 3-day treatment with *G. pictum* extract on total number of hot flushes. SHAM=Sham operated rats treated with the vehicle; OVX=OVX animals treated with the vehicle; E2V=OVX animals treated with estradiol valerate at 1mg/kg BW; GP=OVX animals treated with the aqueous extract of *G. pictum.* (50 and 275 mg/kg BW). ** P <0.01 as compared to control.

After the treatment, during the registration period E2V significantly ($p < 0.01$) reduced the frequency of hot flushes between 18h-00h, 00h-06h and 06h-12h. At the dose tested, *G. pictum* aqueous extract did not significantly reduced the frequency of hot flushes. However, there is a decrease in the frequency of hot flushes between 18h-00h, 00h-06h and 06h-12h on the second day of treatment (Figure.7).

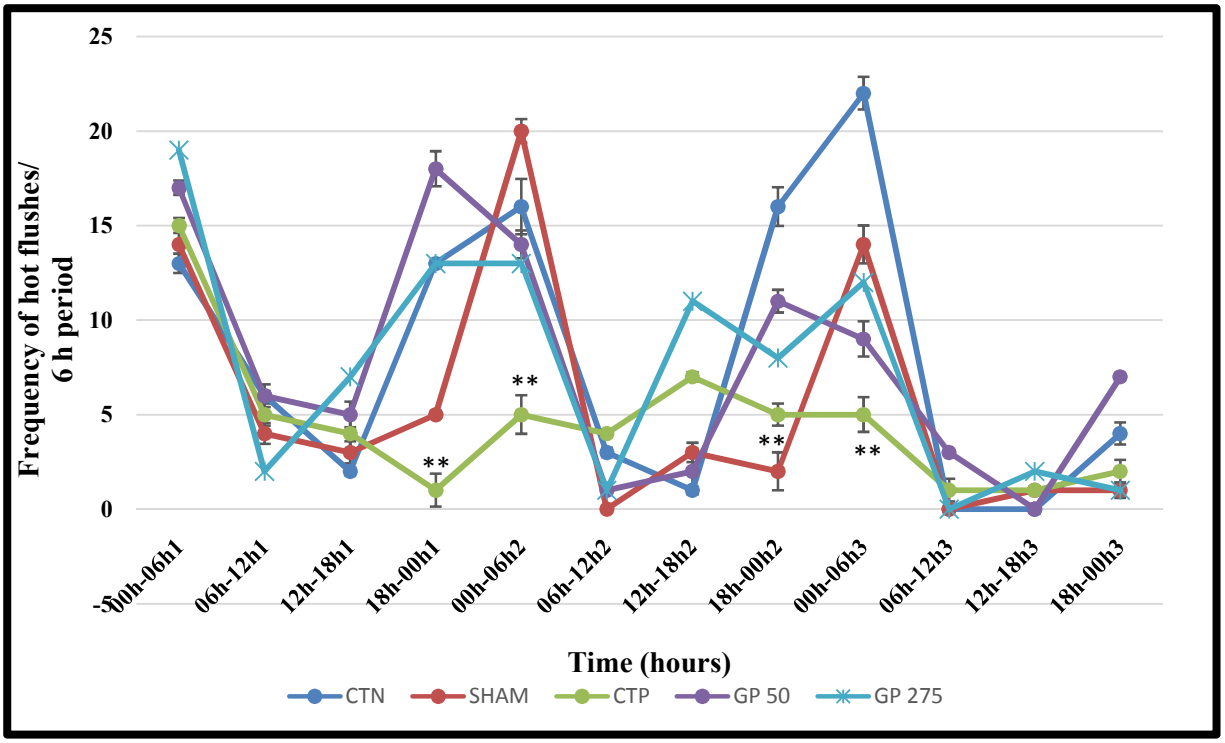


Figure 7: Effects of a 3-day treatment with *G. pictum* extract on frequency of hot flushes. SHAM=Sham operated rats treated with the vehicle; OVX=OVX animals treated with the vehicle; E2V=OVX animals treated with estradiol valerate at 1mg/kg BW; GP=OVX animals treated with the aqueous extract of *G. pictum.* (50 and 275 mg/kg BW). ** P <0.01 as compared to control.

Discussion

Estrogenic effects on the uterus

Increase in the relative uterus weight and the epithelium size, called "uterotrophic" effects is assigned to the proliferation and / or the water imbibition. Indeed, the uterus reacts to changes in serum levels of estrogen and progesterone throughout the estrous cycle in the rat, and following the binding of estrogen on their receptors, two different effects can be observed in the uterus: fluid retention and/or cell proliferation; these effects results in the increase in the uterine weight and thickness of the uterine epithelium [23]. Results on the uterine epithelial high following treatment with E2V were in agreement with the hypothesis of estrogen-stimulation of the endometrial cell proliferation giving rise to a tall columnar epithelium as reported [24] and which results in a marked increase in the uterine epithelial high. Furthermore, these results corroborate those in the literature by which in the absence of estrogen, uterine epithelium is represented by a single layer of cuboidal columnar cells. Under the effect of estrogen, it becomes pseudostratified, with columnar cells [24].

After 3 days of treatment, all the doses of the aqueous extracts we tested did not induce a significant increase in uterine wet weight; meaning the absence of induction of the imbibition of water by our extracts. As far as the uterine epithelium thickness is concerned, a significant increase in the high of the uterine epithelium was induced by the aqueous extract of *T. benthamii* at the dose of 500 mg / kg BW, *G. pictum* at all the tested doses and the mixture of both plants at the doses of 50 and 275 mg / kg BW. These observations support those of Njamen et al (2013) [20] witch show that substances with estrogenic properties affect the uterus by fluid retention and/or cell proliferation, increasing its weight and / or the high of its epithelium. This suggests the presence in the aqueous extracts, of secondary metabolites capable of inducing cell proliferation that leads to the increase in the thickness of the epithelium, by initiating a cascade of genomic reactions after binding to the ER in the uterus (leading to cellular hypertrophy by

protein synthesis). The significant increase in the relative weight of the uterus and the high of its epithelium induced by E2V are reported to be mediated via ER as demonstrated by the lack of uterine stimulation and mitotic growth responses in ERKO mice [25].

Aqueous extracts of *T. benthamii* at doses of 50 and 275 mg / kg BW and the mixture at the dose of 500 mg / kg body weight did not significantly induced an increase in the size of the uterine epithelium. This could be justified by a "dose-dependent" effect [26] or a "down-regulation" [27] or a "desensitization" [26] of the ER involved in the uterotrophic process. In fact, the physiological response of tissue to the binding of a ligand to its receptor depends on the dose of the said ligand. Furthermore, when the concentration of the ligand increases, the number of receptor sites may decrease [27] or the receiver can lose its ability to induce a physiological response following ligand binding [26]. However, although not significant, the effect of the mixture of both *T benthamii* and *G. pictum* at a dose of 500 mg / kg BW remains higher than that obtained in the negative control. Our results also suggest that all the tested extracts might either antagonized the uterine ER or agonized ERβ to decrease uterine wet weight, since ERβ is claimed to mediate ER -antagonistic effects [28]

The activity of the vagina is cyclical and is mediated by sex steroids, like estrogen ([24]. The histological changes of the vaginal epithelium during the menstrual cycle result in proliferation and stratification of the epithelium. 14 days after ovariectomy followed by 3 days of treatment, the vaginal epithelium of the negative control consisted of a layer of squamous cells forming the germinal layer. The E2V has increased the high of the vaginal epithelium by stimulating proliferation, stratification and cornification of vaginal epithelial cells. Three cell layers were visible as well: the germinal layer, the granular layer and the horny layer. This significant increase of the vaginal epithelium is in accordance with the observations of Buchanan et al (1998) [29] who reported that estrogens consistently stimulate proliferation of the vaginal epithelium leading to the formation of a highly stratified epithelium. This effect is reported to be mediated through the ER [29, 30] as demonstrated by Couse et al. [31] who reported that E2 failed to induce vaginal epithelial proliferation and stratification in ER knockout (ERKO) mice. Conversely, all doses of our extracts did not induce a significant increase in the size of the vaginal epithelium. However, substances with estrogenic properties are capable of increasing the size of the vaginal epithelium by stimulating proliferation, stratification and cornification of vaginal epithelial cells [20]. This has not been the case for our extracts. These results might be explained by the fact that secondary metabolites present in our extracts may have a tissue-specific action. They may have induced selective effects by acting as an agonist of a type of ER (alpha) on the uterus and as an antagonist of the same type of ER (alpha) on the vagina or as second type agonist of ER on said vagina. Indeed, it has been reported that ERβ mediate antiproliferative or antagonistic effects of ER ([28]. In addition, some tissues have a higher number of ER as compared to ERβ, causing thereby a higher affinity of a ligand or agonist for a receptor type over the other. This is the case for the uterus and mammary glands which have a higher number of ER as compared to ERβ ([32].Our findings could then be associated with the down-regulation of ER . In fact, ER is found to be the predominant ER-subtype in rat uterus and vagina [33, 34].

The mammary gland is characterized by the presence, in adult women, of a bistratified acinar epithelium with epithelial and myoepithelial cells, a basement membrane, connective tissue and adipose tissue. In the absence of estrogen, there is a decrease in the lumen of the acini, the absence of eosinophils secretions, and a devolved undifferentiated epithelium [35]. In our study, histological sections of the mammary glands revealed that rats treated with E2V as well as extracts of *T. benthamii* at a dose of 500 mg / kg BW and the mixture of *T. benthamii* and *G. pictum* at the doses of 50 and 275 mg / kg PC, exhibit differentiating acini. Indeed, there is an increase in their light and epithelial proliferation compared to the control group. These results corroborate the findings of Santell et al (1997)[35], and Zingue et al (2013)[18], which have shown that the administration of estrogenic substances inverse regression of the mammary gland induced by ovariectomy. The mammary gland proliferates and the layer of myoepithelial cells differs much from the acinar epithelia [18, 35]. Although less abundant than in the group treated with E2V, we noted the increased presence of eosinophils secretions in the lumen of acini of animals treated with the aqueous extract mixture at a dose of 50 mg / kg BW: Parameter indicator of estrogenicity in the mammary gland [18]; these results support the idea that extracts used in this work may contain secondary metabolites with estrogenic properties.

In a second step, the effect of the two plants mixture was compared to that of each of the plants for each tested dose on the size of the uterine epithelium; this in order to determine the type of interaction between the two plants when combined. Thus, at the dose of 50 mg / kg body weight, the effect of *G. pictum* is similar to that of the mixture of two plants. The administration of *G. pictum* alone at this dose therefore will produce the desired effect upon administration of the mixture: this suggests an "independence" activities between *G. pictum* and *T. benthamii*. [26]. As for the dose of 275 mg / kg body weight, there is a significant difference between the effect of the aqueous extract of the mixture of two plants and that of each of the plants. However, the aqueous extract of *G. pictum* significantly increased the size of the uterine epithelium. This difference in the effect can be due to the increase in dose of *G. pictum*. This suggests once more "independence" of activity at this dose between the two plants [26]. Regarding the dose of 500 mg / kg body weight, there is a significant difference between the effect of the aqueous extract mixture and that of each of the plants. Moreover, the aqueous extract of the mixture of two plants has not increased the size of the uterine epithelium; that has yet been done for the aqueous extract of *T. benthamii* and *G. pictum.* This suggests an interaction "antagonist" between the two plants when mixed with a dose of 500 mg / kg BW. Furthermore, the effect of the aqueous extract of each plant is "dose dependent" [26]. It

increases depending on the dose for *T. benthamii* and and therefore also decreases depending on the *G. pictum.*

Effects of aqueous extracts *Graptophyllum pictum* on Hot flushes

The aqueous extract of *G. pictum* significantly increased the size of the uterine epithelium at a dose of traditional healers (50 mg / kg) whereas *T. benthamii* did not exert the same activity. Therefore, we evaluated the effects of this extract on hot flushes at the dose 50 mg / kg and at the dose of 275 mg / kg. We were thus looking if *G. Pictum* could resolve vasomotor disorders associated with postmenopausal condition: the most characteristic menopausal disorder observed in about 85% of women facing menopause [36].Treatment with estradiol significantly reduced ($p<0.01$) the total number, average duration and frequency of hot flushes increased fourteen days after ovariectomy. These results are in line with assertions according to which hot flushes occur in response to ovariectomy in female mammals and are effectively treated by estrogen replacement [37] or by genistein intake [38, 39]. Although the precise pathophysiology of hot flushes is not yet completely understood, it has been associated with the decline in secretion of ovarian steroids particularly in the free fraction of circulating estradiol [40]. It is also speculated that estrogen increases the size of the thermoneutral zone and raises the sweating threshold thus, reducing the frequency of hot flushes [41, 42]. Indeed, it is believed that hot flushes are caused by the shrinking of the brain's thermoneutral zone [43] Freedman (2001) in such a way that minor changes in core temperature which would otherwise not elicit a thermoregulatory response become sufficient to produce sweating and peripheral vasodilation. Freedman [43] suggested that minor core temperature elevations triggered hot flushes in individuals with reduced or non-existent thermoneutral zones. Concerning the flushing, the aqueous extract of *G. pictum* did not reduce the total number of hot flushes, their average duration and frequency. This can be explained by low affinity of the secondary metabolites *G. pictum* with ER, like the low affinity that phytoestrogens have with ER [40, 44] or their low effects on mechanisms involved in the reduction of hot flashes [28].

Conclusion

This work first of all aimed to provide a scientific base for the traditional use of *Tragia benthamii* Baker (Acanthaceae) and Linn *Graptophillum pictum* (Euphorbiaceae) for the treatment of amenorrhea, dysmenorrhea and widespread infertility; secondly, to assess potential interest against the problems of menopause including vasomotor disturbances. To achieve this, we evaluated the estrogenic properties of aqueous extract of each of these plants and their mixture by a conventional uterotrophic test in Wistar rats. The aqueous extract of *T. benthamii*, the aqueous extract of *G. pictum* and the mixture two plants significantly increased the size of the uterine epithelium. *T. benthamii* and the mixture of the two plants also stimulated the differentiation of acini by increasing their lumen and epithelial proliferation. These actions are likely mediated at the ER by secondary metabolites with estrogenic properties contained in our plants. The aqueous extracts of our plants did not induce a significant increase in uterine weight and the size of the vaginal epithelium. The size of the uterine epithelium was not modified either by *T. benthamii*, or by the mixture of two plants. Given the results we obtained, it appears that *T. Benthanii* and *G. pictum* have a weak but observable oestrogen-mimetic potential, justifying their traditional use against secondary amenorrhea in women.

Authors' contributions

The idea of the study was conceived by Ketcha Wanda and Djoussi Njimfo, the experimental work and analysis of the data generated was done by Awounfack and Djoussi. the manuscript was prepared by Ketcha wanda and Djiogue. Njamen participated in the conception of the study, its design and coordination and helped to draft the manuscript.

Acknowledgment

The authors would like to thank Mr. Tebo Ndifon Emmanuel for the critical reading.

Conflict of interest

All the authors state that there are no conflicts of interest within this article.

References

[1]. Böttner M, Christoffe I J, Jarry H, Wuttke W. Effects of long term treatment with resveratrol and subcutaneous and oral estradiol administration on pituitary function in rats. J Endocrinol 2006; 189: 77–88.

[2]. Pinkerton JV, Stovall DW, Kighlinger RS. Advances in the treatment of menopausal symptoms. Womens Health 2009; 5: 361–84.

[3]. Nkwo, P, Onah H. Positive attitude to menopause and improved quality of life

among Igbo women in Nigeria. Int J Gynecol Obstet 2008; 103: 71–72.

[4]. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML. Writing Group for the Women's Health Initiative Investigators, 2002. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. J Am Med Assoc 2003; 288: 321–333.

[5]. Million Women Study Collaborators. Breast cancer and hormone replacement therapy in the Million Women Study. Lancet 2003; 362: 419–427.

[6]. Million Women Study Collaborators. Endometrial cancer and hormone-replacement therapy in the Million Women Study. Lancet. 2005;365: 1543–1551.

[7]. Brzezinski A, Debi A. Phytoestrogens: the "Natural" selective estrogen receptor modulators? Eur J Obstet Gynecol Reprod Biol 1999;85:47–51.

[8]. Pitkin, J., 2012. Alternative and complementary therapies for menopause. Menopause Int 2012; 18: 20–27.

[9]. Kaufert P, Boggs B, Ettinger NF, Woods WH. Women and menopause: beliefs, attitudes and behaviors. The North American Menopause Society 1997 Menopause Survey, Menopause 1998; 5: 197–202.

[10]. Ferrari A. Soy extract phytoestrogens with high dose of isoflavones for menopausal symptoms. J Obstet Gynaecol Res 2009 ;35: 1083–90.

[11]. Bouquet A, Debray. Féticheurs et plantes médicinales de la Cote d'Ivoire. Office de la Recherche Scientifique et Technique d'Outre-mer (ORSTOM), Paris 1974 ; pp. 71.

[12]. Jiofack T, Fokunang C, Guedje N, Kemeuze V, Fongnzossie E, Nkongmeneck BA, Mapongmetsem PM, Tsabang N. Ethnobotanical uses of medicinal plants of two ethnoecological regions of Cameroon. Intl J of Med and Medical Sciences 2010;2(3) pp. 60-79.

[13]. Perry LM. Medicinal plants of East and South-East Asia; Attributed Properties and Uses MIT press, Cambridge, United States and London 1980;p. 2.

[14]. Ozaki Y, Sekita S, Soedigdo S, Harada M. (1989), Anti-inflammatory effect of *Graptophyllum pictum* (L.) GRIFF. Chemical pharmaceutical bulletin 1989;37(10): 2799-802.

[15]. Stella OOD, Grace EU, Herbert ABC, Samuel AA. Oxytocic and anti-implantationactivities of the leaf extracts of Graptophyllum pictum (Linn.) Griff. (Acanthaceae). AfricanJournal of Biotechnology 2009; 8(21):5979-84.

[16]. Olagbende-Dada SO, Ogbonnia SO, Coker HAB, Ukpo GE. Blood glucose lowering effect of aqueous extract of Graptophyllum pictum (Linn) Griff. On alloxan-induced diabetic rats and its acute toxicity in mice. African Journal of Biotechnology 2011;10(6) 1039-1043.

[17]. Lane NE,Yao W,Kinney JH,Modin G, Balooch M, Wronski TJ,2003.Both hPTH (1-34)and bFGF increase trabecular bone mass in osteopenic rats but they have different effects on trabecular bone architecture. J Bone Miner Res. 2003; 18: 2105–2115.

[18]. Zingue S, Njamen D, Tchoumtchoua J, Halabalaki, M, Simpson, E, Clyne C, Magne Nde CB.Effectsof Millettia macrophylla (Fabaceae) extracts on Estrogen target organs of female Wistar rat. J Pharmacol Sci 2013; 123: 120–131.

[19]. Berendsen HHG, Weekers AHJ, Kloosterboer HJ.Effect of tibolone and Raloxifene on the tail temperature of estrogen-deficient rats. Eur J Pharmacol 2001; 419:47–54.

[20]. Njamen D, Djiogue S, Zingue S, Mvondo MA, Nkeh-Chungag BN 2013. In vivo and in vitro estrogenic activity of extracts from Erythrina poeppigiana (Fabaceae). J Complement Integr Med 2013; 10:1–11.

[21]. Nkeh-Chungag BN, Tiya S, Mbafor JT, Ndebia EJ, Rusike S, Iputo JE. Effects of the methanol extractof Erythrina abyssinica on hot flashes in ovariectomized rats. Afr J Biotechnol 2013; 12: 598–601.

[22]. Maswood, Cosmi S, Alfinito PD, Leventhal L, Deecher DC. The role of the selective serotonin reuptake inhibitor fluoxetine in temperature regulation in ovariectomized rat models. Neuroendocrinology 2006; 84: 330–338.

[23]. O'Brien JE, Peterson TJ, Tong MH, Lee EJ, Pfaff LE, Hewitt SC, Korach KS, Weiss J Jameson JL. Estrogen-induced prolifération of uterine epithelial cells is independent of estrogen receptor binding to classical estrogen response elements. J Biol Chem 2006; 281: 26683–26692.

[24]. Westwood FR. The female rat reproductive cycle: a practical histological guide to staging. Toxicol Pathol. 2008;3(6):375–84.

[25]. Hewitt SC, Deroo BJ, Hansen K, Collins J, Grissom S, Afshari CA, Korach KS. Estrogen receptor-dependent genomic responses in the uterus mirror the biphasic physiological response to estrogen. Mol Endocrinol 2003;17:2070–83.

[26]. Rang HP, Dale MM, Ritter JM, Moore P. K. Pharmacology. Churchill Livingstone, Elsevier science limited, 2003; 11-20.

[27]. Couse JF, Korach KS. Contrasting phenotypes in reproductive tissues of female estrogen receptor null mice. Ann NY Acad Sci. 2001; 948: 1-8.

[28]. Weihua Z, Andersson S, Cheng G, Simpson ER, Warner M, Gustafsson JA. Update on estrogen signalling. FEBS Lett 2003; 546: 17–24.

[29]. Buchanan DL, Kurita T, Taylor JA, Lubahn DB, Cunha GR, Cooke PS. Role of stromal and epithelial estrogen receptors in vaginal epithelial proliferation, stratification, and cornification. Endocrinology. 1998;139:4345–52.

[30]. Buchanan DL, Setiawan T, Lubahn DB, Taylor JA, Kurita T, Cunha GR, Cooke PS. Tissue compartment-specific estrogen receptor-a participation in the mouse uterine epithelial secretory response. Endocrinology 1999;140:484–91.

[31]. Couse JF, Curtis SW, Washburn TF, Lindzey J, Golding TS, Lubahn DB, Smithies O, Korach KS. Analysis of transcription and estrogen insensitivity in the female mouse after target disruption of the estrogen receptor gene. Mol Endocrinol. 1995;9:1441–54.

[32]. Dahlman-Wright K, Cavailles V, Fuqua SA, Jordan VC, Katzenellenbogen JA, Korach KS,Maggi A, Muramatsu M, Parker MG, Gustafsson JA. International Union of Pharmacology. LXIV. Estrogen receptors. Pharmacol Rev 2006; 58: 773–781.

[33]. Pelletier G, Labrie C, Labrie F. Localization of oestrogen receptor alpha, oestrogen receptor beta and androgen receptors in the rat reproductive organs. J Endocrinol 2000;165:359–70

[34]. Wang H, Eriksson H, Sahlin L. Estrogen receptors alpha and beta in the female reproductive tract of the rat during the estrous cycle. Biol Reprod. 2000;63:1331–40

[35]. Santell RC, Chang YC, Nair MG Helferich WG. Dietary genistein exerts estrogenic effects upon the uterus, mammary gland and the hypothalamic / pituitary axis in rats. J Nutr 1997;127: 263–269.

[36]. Koang MP. Traitement des bouffées de chaleur par acupuncture. Meridien 1992 95: 121–140.

[37]. Santoro N, 2008.Symptoms of menopause:hot flushes. Clin Obstet Gynecol 2008; 51:539–548.

[38]. Crisafulli A, Marini H, Bitto A , Altavilla D, Squadrito G, Romeo A, Adamo EB, Marini R, D'Anna R, Corrado F, Bartolone S, Frisina N, Squadrito F. Effects of genistein on hot flushes in early postmenopausal women: a randomized, double-blind EPT-and placebo-controlled study. Menopause 2004; 11: 400–404.

[39]. D'Anna R, Cannata ML, Marini H, Atteritano M, Cancellieri F, Corrado F, et al.,Effects of the phytoestrogen genistein on hot flushes, endometrium, and vaginal epitheliumin post-menopausal women: a 2-year randomized, double-blind, placebo-controlled study, Menopause 2009; 16 301–306.

[40]. Erlik Y, Meldrum DR, Judd H. Estrogen levels in post menopausal women with hot flashes, Obstet Gynecol. 1982; 59:403–407.

[41]. Freedman RR, Blacker CM. Estrogen raises the weating threshold in postmenopausal women with hot flashes. Fertil Steril 2002; 77: 487–490.

[42]. Dacks PA, Rance NE. Effects of estradiol on the thermoneutral zone and core temperature in ovariectomized rats, Endocrinology. 2010; 51: 1187–1193.

[43]. Freedman RR. Physiology of hot flashes. Am. J. Hum. Biol.2001;13,4553–4564.

[44]. Le Bail JC, Champavier Y, Chulia AJ, Habrioux G. Effects of phytoestrogens on aromatase, 3beta and 17beta-hydroxysteroid dehydrogenase activities and human breast cancer cells. Life Sci 2000; 66: 1281–1291.