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Gouania Longipetala Aqueous Extract Exhibits Antiestrogen Activities and Activate Estrogen Receptors in Ovariectomized Wistar Rats

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ABSTRACT

Previous studies showed that *G. longipetala* aqueous extract (GLAE) induced estrogenic properties in ovariectomized (Ovx) rats. The present study aimed to assess antiestrogenic activities and signaling pathways of GLAE. For that, two pharmacological tests were used to evaluate GLAE effects in ovariectomized rats treated with estradiol valerate (E_2V) and a pure antiestrogen (ICI 182.780). Animals were either sham-operated or Ovx. 3-day uterotrophic assay was carried out in Ovx adult Wistar rats and GLAE effects were evaluated on the genital tract and mammary gland. Results showed that GLAE reduced significantly vaginotrophic and mammotrophic effects of estradiol valerate. Indeed, GLAE reduced by 68.72% and 62.66% respectively for 45 and 180 mg/kg the vaginal epithelial height of E_2V-Ovx rats. Besides, ICI 182.780 cancelled estrogenic activities of GLAE in both genital tract and mammary gland. Overall, *Gouania longipetala* aqueous extract activates estrogen receptors to induce estrogen activities and exhibit antiestrogenic properties.

KEYWORDS: *Estrogenic receptors, ovariectomized, ICI 182.780, estradiol valerate, Gouania longipetala*

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INTRODUCTION

To improve the condition of menopausal women and to treat the pathologies that appear during this unavoidable period of a woman's life, there are several effective hormonal and non-hormonal approaches [1,2]. Hormone replacement therapy (HRT), based on estrogen with or without progesterone, is widely used [3]. However, it is expensive, sometimes unaffordable, and adverse side effects such as an increased risk of hormone-dependent cancer make women increasingly reluctant to use this treatment [4]. Selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene while effective, also have deleterious effects [5,6]. Therefore, the search for new molecules with estrogenic properties and limited adverse effects is underway [7]. Nature is an inexhaustible source of molecules with biological activities [8]. Approximately 70% of the drugs on the market today are of natural origin [9]. In addition, tribal and religious beliefs promote the use of herbal medicine, especially in developing countries [10].

Phytoestrogens are non-steroidal compounds of plant origin that mimic the effects of estrogens or inhibit the effects of androgens [11]. Studies show that these compounds have beneficial effects on cardiovascular disease, vaginal dryness, hot flashes, and even neurodegenerative diseases [12,13]. They are considered to be a safer HRT, hence the considerable increase in their consumption in the form of dietary supplements. However, clinical data remain limited and the risk of triggering or aggravating estrogen-dependent cancers is not satisfactorily known. Insofar as phytoestrogens have multiple effects, it appears interesting to

elucidate their different properties and the doses that should be prescribed [14,10].

The Rhamnaceae includes about 900 plant species, including *Gouania longipetala*, which is the subject of this study [15]. Ethnobotanical uses of *G. longipetala* include treatment of worms, abdominal pain, lumbago, ophthalmia, rickets, gout, venereal disease, and stomach ache among others. Anti-inflammatory, antioxidant, and antibacterial effects of *G. longipetala* trunk bark have been reported in Ghana [16-18]. In Cameroon, *Gouania longipetala* known locally as Sobomissile in Badjoue or Alamawaso'o in Bamumbu, is used to treat female reproductive problems [19]. Previous studies showed that both aqueous and extract of *G. longipetala* exhibit estrogenic properties in ovariectomized rats [20]. Thus, this study aimed to evaluate the probable pathways used by aqueous extract in ovariectomized rats. For that, GLAE effects were assessed in ovariectomized rats treated with estradiol valerate and a pure antiestrogen (ICI 182.780).

Material and methods

Chemicals

Estradiol valerate (E_2V) marketed as Progynova® 2 mg was supplied by Delpharm (Lille, France). The fulvestrant (ICI 182.780) marketed under the name of Falslodex® was supplied by Tocris Biosciences (Bristol, UK).

Plant material

After shade-drying, barks of *G. longipetala* (GLAE) were crushed into a fine powder. The aqueous extract was prepared according to the method used in traditional medicine. Indeed, 100 g of the obtained bark powder was mixed with 3 L of tap water, and the whole was boiled for 30 minutes. After cooling, the decoction obtained was filtered with Whatman number 3 and the filtrate obtained was evaporated in an oven at 45 °C.

Animal material

Animals used were female albino rats (*Rattus norvegicus*) of Wistar strain aged 8 to 11 weeks weighting 100-130 g. They were reared in plastic cages at the Animal Physiology Laboratory of the University of Yaoundé I. Reared conditions were: room temperature, normal day/night alternation, free access to tap water and soy-free food. All experiments were conducted in accordance with the principles and procedures of the European Union on Animal Care (CEE Council 86/609) guidelines adopted by the Cameroon Institutional National Ethic Committee, Ministry of Scientific Research and Technology Innovation (Reg. number FWA-IRD 0001954).

Experimental design

Animals were either sham-operated or ovariectomized (Ovx). For the first pharmacological test aimed to evaluate anti-estrogenic properties of GLAE, Ovx animals were divided into 4 batches of 5 rats each and received respectively distilled water (10 mL/kg), estradiol valerate (1 mg/kg), GLAE at 45 and 180 mg/kg combined to estradiol valerate (E_2V). For the second pharmacological test aimed to determine the effects of ICI 182.780on the activities of aqueous extract of *G. longipetala*, 40 ovariectomized rats were randomized into 2 groups. The first group consisted of 5 batches: the first batch was sham-operated and received distilled water; the remaining batches were ovariectomized and then these batches received distilled water (10 mL/kg), estradiol valerate (1 mg/kg), and the aqueous extract of *Gouania longipetala* at doses of 45 and 180 mg/kg, respectively. The second group also consisted of 5 batches that received the aforesaid treatment combined with ICI 182.780at a dose of 250 µg/kg. The substances were administered orally after 14 days, except for ICI 182,780, which was administered subcutaneously.

The treatment was performed by gavage with an esophageal tube and lasted three days, after which the rats underwent a vaginal smear and were then sacrificed. The uterus was removed and weighed. One uterine horn was fixed in Bouin's fluid and the other was homogenized at 20% for protein determination. The nipple and vagina were removed and fixed in Bouin's fluid for histological analysis.

Vaginal cellular differentiation

Evaluation of vaginal cornification was conducted using the protocol of [21], a method that is based on the observations of Stockard and Papanicolaou. 0.5 mL of 0.9% NaCl was introduced in the vagina and vaginal cells were collected and deposited on a slide using a pipette. 10 µL of these vaginal contents were collected with a micropipette and spread on a rimmed slide. Once the samples were spread, they were then stained according to the Papanicolaou method.

Determination of uterine relative weight

The relative fresh weight of uterus, abdominal fat and aorta was calculated using the following formula according to [22]:

$$\text{Organ weight ratio} = \frac{\text{Uterus weight (g)}}{\text{Body weight (g)}} \times 100$$

Histomorphometric analysis of uterine, vaginal, and mammary gland tissues

Uterine, vaginal, and mammary gland tissues after fixation (2 weeks) in Bouin's liquid were trimmed and dehydrated in alcohol of croissant gradient (70 %, 80 %, 90 % and 100 % (3 baths)). After tissues were clarified in 2 baths of xylene (1h30min per bath) and impregnated in liquid paraffin at 60 °C (for 5 hours). Uterine, vaginal, and mammary gland epithelial sizes were assessed from 5 µm sections of paraffin-embedded and haematoxylin–eosin or Masson's trichrom stained. Epithelial sizes were assessed on microphotographies obtained by using a light microscope (Leitz wetzlar Germany 513) connected with a digital camera DCM35, 350K pixels, USB2.0 connected to a computer where images were transferred and analyzed using Image J software.

Statistical analysis

Data were expressed as mean ± standard error on mean. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test using GraphPad Prism 8.0.1. A value of $p < 0.05$ was considered statistically significant.

Results

1. Effects of combined administration of GLAE and E_2V in 3-day Ovx rats

1.1 Effects on vagina

Once-daily administration of E_2V for three days induced the increase of expression of superficial secretory epithelial cells compared with Ovx animals receiving distilled water. Indeed, the number of superficial epithelial cells increased from 6.67 ± 1.83 in Ovx animals receiving distilled water to 3626.67 ± 523.56 in Ovx animals treated with E_2V. This preponderance of superficial cells on the vaginal smear of E_2V-treated Ovx rats was reflected with the appearance of *stratum granulosum* and *stratum corneum* in the vaginal epithelium. In rats receiving both E_2V and GLAE, a reduction of vaginal differentiation was observed. The reduction of the number of superficial epithelial cells was about 38.07% and 45.48% at 45 and 180 mg/kg of GLAE respectively. The same doses reduced the size of the vaginal epithelium from 62.69 ± 3.41 µm in E_2V-treated animals to 46.43 ± 1.89 µm and 32.16 ± 1.55 µm respectively.

	Cells/µL			Estral cycle	Epithelial Height
Lots	**Parabasal**	**Intermediate**	**Superficials**		
Sham	55.98 ± 25.1	402.34 ± 48.2	1836.95 ± 78.7	**Estrus**	54.37 ± 2.56
Vehicle	135.33 ± 13.05^{h}	/	6.67 ± 1.83^{z}	**Diestrus**	16.37 ± 1.33^{z}
E_2V	/	453.33 ± 72.36	3626.67 ± 523.56^{zc}	**Estrus**	62.69 ± 3.41^{zc}
GLAE45 + E_2V	/	641.50 ± 36.77$^{\delta}$	2246.00 ± 155.46$^{zc\delta}$	**Estrus**	46.42 ± 1.89$^{c\delta}$
GlAE180 + E_2V	/	580.00 ± 60.38$^{\beta h}$	1984.67 ± 88.49$^{c\delta i}$	**Estrus**	32.15 ± 1.54$^{c\delta}$

Table I: Effects of GLAE in E_2V-treated ovariectomized rat on vaginal cells **1**: *stratum germinativum*, **2**: *stratum granulosum*, **3**: *stratum corneum*, **4**: lumen.

Values represent the mean ± SEM (n = 5).$^{h}p < 0.01$: significant difference from sham batch.$^{c}p < 0.001$: significant difference from control receiving the vehicle; $^{\beta}p < 0.01$, $^{\delta}p < 0.001$: significant difference from animal treated with estradiol valerate.

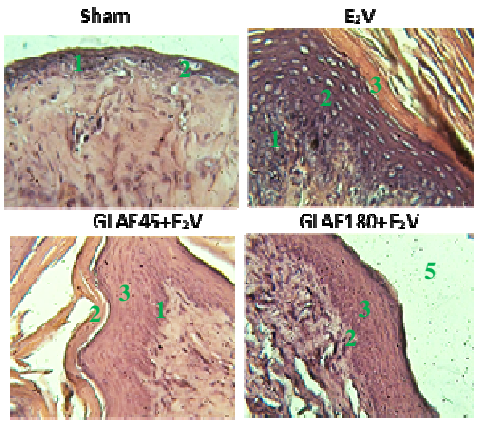

Figure 1: Effects of GLAE in E_2V-treated ovariectomized rat on vaginal epithelium (HE Staining)

1.2 Effects on uterine tissue

Exclusive treatment of ovariectomized rats with estradiol valerate resulted in a significant increase in uterine protein level 82.35% ($p < 0.01$) and relative uterine weight 406.35% ($p < 0.001$) compared to the control receiving water. The administration of GLAE administration in estradiol valerate treated rats resulted in a significant ($p < 0.001$) and dose-dependent reduction in uterine protein levels (Figure 2B) and relative uterine weight (Figure 2A) compared to the control. Estradiol valerate induced the differentiation of cubic uterine epithelial cells in a stratified cylinder epithelium in Ovx animals (Figure 2C). This induced a 195.39% increase in the epithelial height of the uterus. GLAE reduced significantly E_2V effects on the uterine epithelium. This reduction

was by 68.72% and 62.66% respectively for 45 and 180 mg/kg of GLAE (Figure 2D).

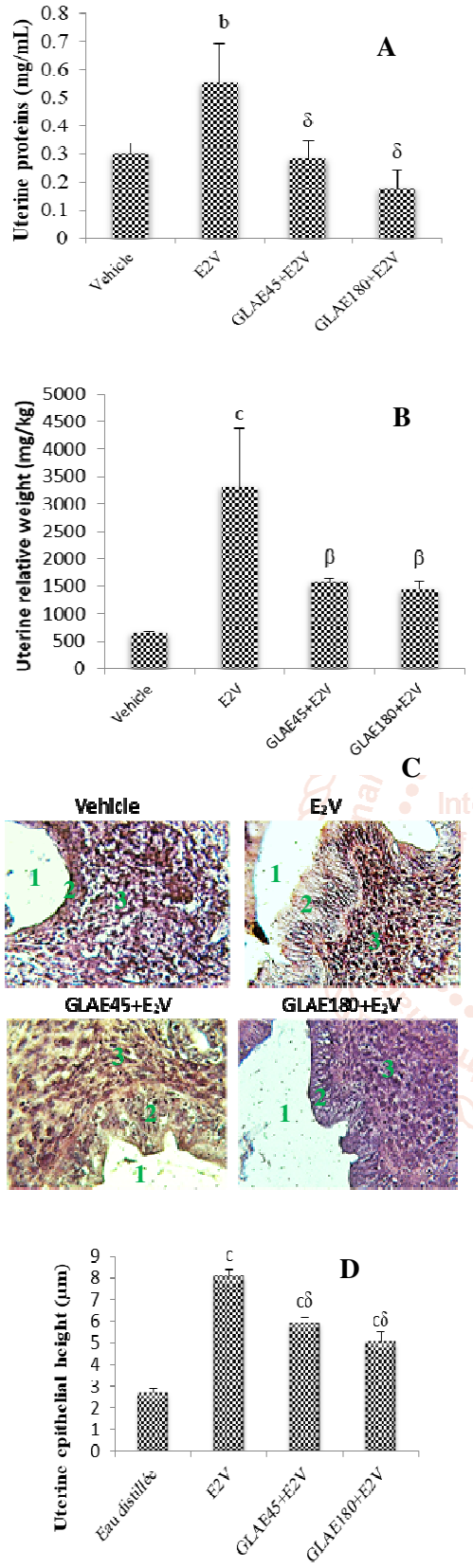

Figure 2: Effects of GLAE in E_2V-treated ovariectomized rat on uterine weight and epithelium (HE staining, x 400)

Values represent the mean ± SEM (n = 5). $^{b}p < 0.01$ $^{c}p < 0.001$: significant difference from control receiving the vehicle; $^{\beta}p < 0.01$, $^{\delta}p < 0.001$: significant difference from animal treated with estradiol valerate. **1**: lumen; **2**: uterine epithelium; **3**: *lamina propria*

1.3 Effects on the mammary gland

Estradiol valerate induced the development of acinar epithelium as well as the production of eosinophilic secretions in the lumen of the acini compared to the Ovx control receiving distilled water (Figure 3A). This development of the ductile epithelium is characterized by a 24.10% increase in its height compared to the Ovx control. GLAE at 180 mg/kg induced a significant decrease of the acinar and epithelium heights compared to E_2V-treated Ovx rats. Regarding the size of the acinar epithelium, it decreased by 1.53- fold (Figure 3B).

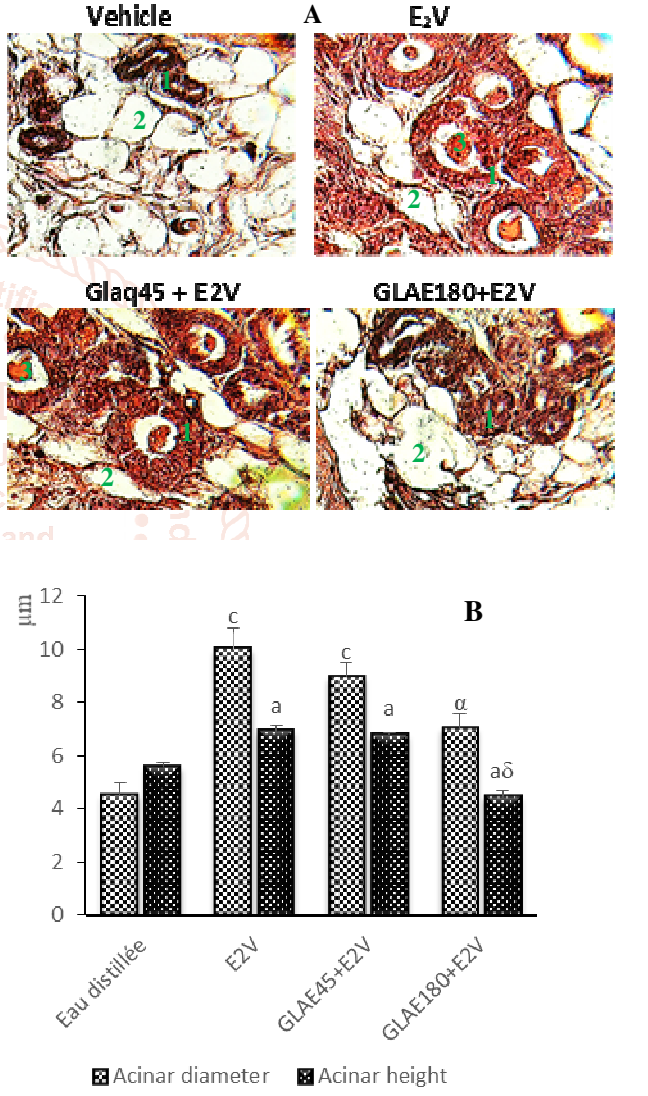

Figure 3: Effects of GLAE on mammary gland of E_2V-treated ovariectomized rat (HE staining x400)

Values represent the mean ± SEM (n = 5). $^{b}p < 0.01$ $^{c}p < 0.001$: significant difference from control receiving the vehicle; $^{\beta}p < 0.01$, $^{\delta}p < 0.001$: significant difference from animal treated with estradiol valerate. **1**: acinar epithelium; **2**: adipose tissue; **3**: eosinophil secretion.

2. Effects of ICI 182.780 on GLAE estrogenic activities

2.1 Effects on vaginotrophic activities

The administration of ICI 182.780 in 14-day Ovx rats resulted in the inhibition of effects of both E_2V and GLAE. Indeed, ICI 182.780induced the disappearance of *stratum granulosum* in animals treated with the plant extract compared to controls treated exclusively with GLAE. Indeed, GLAE induced a reduction in the vaginal epithelial size of 33.62 and 60.33% in Ovx rats receiving the aqueous extract at doses of 45 and 180 mg/kg, respectively, compared to rats treated with the extract alone. Under the same conditions, ICI 182.780induced a 70.57% reduction in vaginal epithelial size in Ovx rats treated with estradiol valerate.

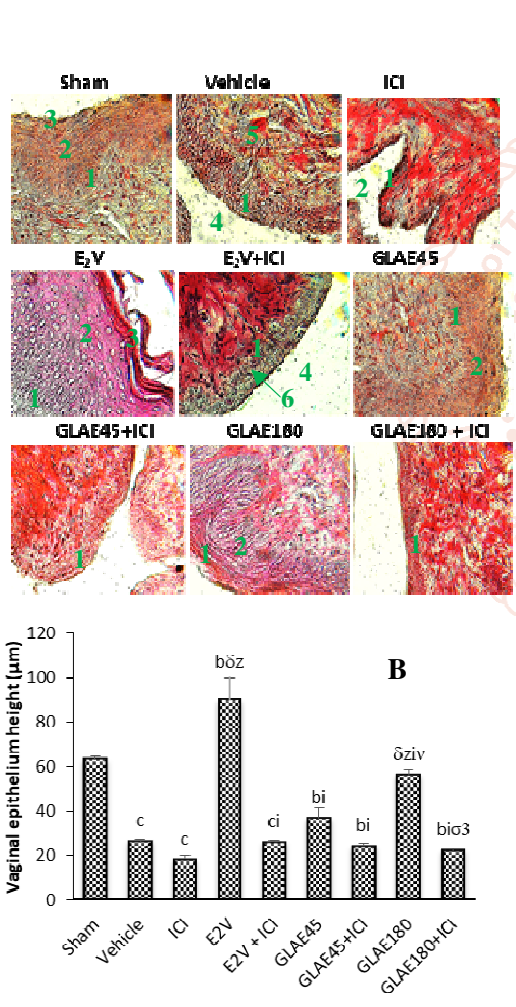

Figure 4: Effects of ICI 182.780 on GLAE vaginotrophic activities (Masson's trichrom, x 400)

Bars represent the mean ± S.E.M (n = 5). $^{b}p < 0.01$, $^{c}p < 0.001$: significant difference from the Sham control; $^{\delta}p < 0.001$: significant difference from the control receiving distilled water; $^{z}p < 0.001$: significant difference from the control receiving ICI 182.780; $^{i}p < 0.001$: significant difference from control receiving E_2V; $^{v}p < 0.05$, $^{\sigma}p < 0.001$: significant difference from GLAE45 batch, $^{3}p < 0.001$ significant difference from GLAE180 batch. **1**: *stratum germinativum*; **2**: *stratum granulosum*; **3**: *stratum corneum*; **4**: *lamina propria*; **5**: *lumen*; **6**: *necrosis*.

Effects on uterotrophic activities

Oral administration of ICI 182.780 at a dose of 250 µg/kg reduced uterine weight by 1.30-fold and uterine protein levels by 22.53% in ovariectomized rats compared with the untreated Ovx control. Fourteen days after ovariectomy, treatment of rats with aqueous extract of *G. longipetala* alone or concomitantly with pure antiestrogen did not significantly change uterine weight and uterine protein level compared to controls. Administration of estradiol valerate alone to Ovx rats resulted in a significant ($p < 0.001$) increase in relative uterine weight and uterine protein level. This increase was reduced by 50.90 and 63.36%, respectively, in the presence of pure anti-estrogen (Figure 4A, Figure 4B).

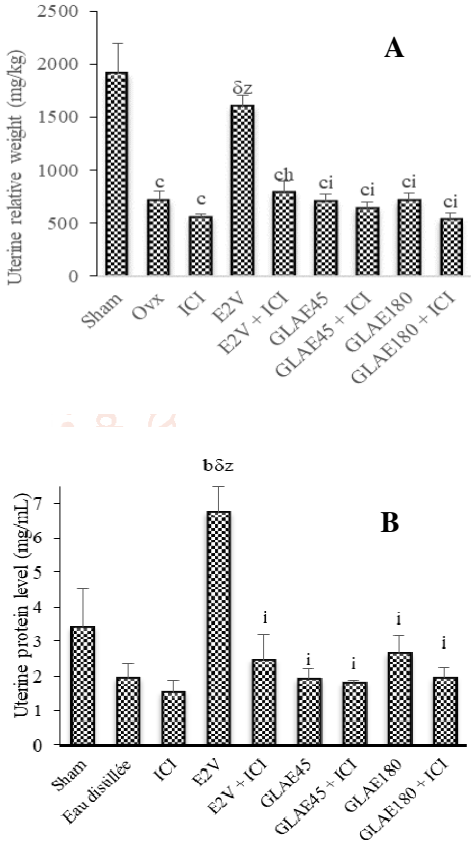

Figure 4: Effects of ICI 182.780 on GLAE uterotrophic activities

Bars represent the mean ± S.E.M (n = 5). $^{b}p < 0.01$, $^{c}p < 0.001$: significant difference from the Sham control; $^{\delta}p < 0.001$: significant difference from the control receiving distilled water; $^{z}p < 0.001$: significant difference from the control receiving ICI 182.780; $^{i}p < 0.001$: significant difference from control receiving E_2V.

Discussion

Plant-based diets as well as their consumption has increased worldwide. Phytoestrogens, are compounds with similar structures or/and function with endogenous estrogen. Well-known for their antioxidant properties, many proofs from in vivo studies suggest that they may have a huge impact on hormones and health [10]. Their structure allow

them to bind to both estrogen receptors (ER), ERα, and ERβ, exhibiting a weak estrogenic activity [23]. In the present study, exclusive treatment of ovariectomized animals with estradiol valerate (E_2V) resulted in a vaginal expansion of the *stratum granulosum* and keratinization of superficial cells into *stratum corneum*. This E_2V-induced differentiation was significantly reduced by treatment of the animals with the aqueous extract of *Gouania longipetala* (GLAE). These results suggest that *G. longipetala* extract contains phytoestrogens like flavonoids reported in a previous study [20] that are able to compete with 17β-estradiol for its receptor. The same result is observed both in the uterine and mammary gland. Thus, GLAE exhibits anti-estrogenic properties and then can be useful for hormone-dependent cancers (breast and endometrial) in postmenopausal women [23]. Indeed, while stimulating ERs, estrogen-like compounds contains in plant are able to activate the transcription of several target genes. This results in the increase of the levels of antioxidants enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase [25,26]. Previous studies also reported the activation of antioxidants enzymes by GLAE [16,20].

The hypothesis that the metabolites contained in GLAE act via the ERs was confirmed by the assay with ICI 182,780. Indeed, the results of the present work showed that ICI 182.780 completely inhibited the vaginotrophic effects of the aqueous extract of *Gouania longipetala*. Pure anti-estrogens, such as ICI 182,780, are able to reduce estrogen receptor levels by inactivating them [27]. The antiproliferative action of GLAE also extends to the uterine and acinar epithelia. Concomitant treatment of ovariectomized rats with estradiol valerate and aqueous extract of *G. longipetala* significantly reduced the relative weight of the uterus and the uterine protein level, whereas the extract alone had no effect on these parameters. This effect would be due to the inhibitory action of flavonoids contained in the extract on the activation of transcription factors. Virgili et al. [28] reported that flavonoids deteriorated the interaction between ER and the transcription factors Sp1 and AP-1 in the presence of estrogens. The antiproliferative action of plant extract could also be due to catechins present in GLAE [20] whose antiproliferative effects were reported [29].

Conclusion

As a result of pharmacological studies, *Gouania longipetala* activates estrogen receptors and induced anti-estrogenic activities. Thus, it confirmed the traditional uses of *G. longipetala* against menopausal complaints. Furthermore, the anti-estrogenic properties of the plant extract may be benefic for estrogen dependent cancers.

Glossary

E_2V: Estradiol valerate or batch of Ovx animals treated with estradiol valerate (1 mg/kg)

ERs: Estrogen receptors

GLAE: *Gouania longip*etala aqueous extract

GLAE45+E_2V,GLAE180+E_2V: batches of Ovx animals and concomitantly treated with the aqueous extract of *G. longipetala* at doses 45 and 180 mg/kg and estradiol valerate.

ICI 182.780: pure antiestrogen (fulvestrant)

Ovx: Ovariectomized

Sham: lot of non-ovariectomized animals given distilled water (10 mL/kg);

Vehicle: lot of ovariectomized (Ovx) animals receiving distilled water (10 mL/kg)

Conflicts of Interest

Authors declare that there is no conflict of interest.

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Author's contribution

MNYS carried out the experiments, histological and biochemical analysis. DDPD designed the study.

All the authors were involved in the draft and review of the manuscript.

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