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In vitro and *in vivo* antiestrogenic effects of dichloromethane-methanol extract of *Crateva adansonii* DC.

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Despite significant developments occurring in the treatment of cancer, it still remains the second deadly disease, responsible for 8.2 million deaths every year. Various natural substances have been studied for active molecules of tumor suppression in the past and the tropical flora, by its diversity, continues to provide new antitumor drugs. *Crateva adansonii* dichloromethane-methanol (DCM/MeOH) extract was previously reported to prevent breast tumors in Wistar rats. However, it exhibited weak cytotoxic effect in human MCF-7 cells. The present study, therefore, deals with the investigation of its estrogenic and antiestrogenic effects. *In vitro* estrogenicity and antiestrogenicity of *C. adansonii* DCM/MeOH extract were performed by E-screen assay. *In vivo*, the investigation was carried out using the 3 days uterotrophic assay in ovariectomized rats, a classical tool for the prediction of estrogenicity of chemicals. As a result, *C. adansonii* extract did not induce MCF-7 cells proliferation, which is an estrogenic hallmark. However, *C. adansonii* extract induced a significant (P <0.05) decrease in a concentration-dependent manner of the MCF-7 proliferation when co-administered with E2B. *In vivo*, no estrogen-like effect was observed following a 3-day treatment with *C. adansonii* extract in estrogen target organs. However, the co-administration of *C. adansonii* extract with E2V lead to decreased uterine wet weight (P <0.05), total protein levels in uteri (P <0.01) as well as uterine and vaginal epithelial heights (P <0.05) as compared to animals treated with E2V only. These results suggest that *C. adansonii* has antiestrogenic effects but not estrogenic effects, which might account for its previously observed antimammary tumour effects in rats.

Keywords: Antiestrogenic, *Crateva adansonii*, E-screen assay, Uterotrophic assay

Estrogens are steroid hormones involved in almost all metabolisms (brain, cardiovascular system, kidneys, immune system, liver and bone) in women with an important function in the regulation of sex process[1,2]. Due to its ubiquitous distribution and proliferative effects, estrogens play an important role in the development and progression of estrogen dependent cancers. Cancer can be defined as a group of diseases characterized by the unregulated proliferation of abnormal cells that invade and disrupt surrounding tissues[3]. It is a source of significant morbidity worldwide, responsible for over 8.2 million deaths per year and a serious public health problem in both, developed and developing countries[4]. In developing countries including Cameroon, estrogen-dependent cancers (breast, uterus, and ovarian) account for 19% of cancers worldwide and are among the leading causes of morbidity and mortality in these countries[5].

Blockage of estrogens production with specific inhibitors of steroidogenic enzymes such as aromatase inhibitors is one of the two currently available standard therapies. The other approach involves blocking the estrogen receptor (ER) with selective estrogen receptor modulators (SERMs) such as tamoxifen[6,7]. Although these therapies are effective to treat and/or prevent estrogen-dependent cancers, their long term use is associated with side effects and the therapy only reduces the risk of recurrence by 30-50%[8]. Therefore, non-synthetic and natural

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Abbreviations: CNH, Cameroon National Herbarium; DCM, dichloromethane; E2V, estradiol valerate; ER, estrogen receptor; ERα, estrogen receptor alpha; ERβ , estrogen receptor beta; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; MeOH, metanol; OVX, bilaterally ovariectomized rats; RPMI, Roswell Park Memorial Institute; SERM, selective estrogen receptor modulators; SEM, Standard Error of Mean; SRB, sulforhodamine-B

alternative, that might 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 dependent tissues, such as bones, brain and liver are needed; and phytoestrogens are promising candidates[9]. Phytoestrogens are plant metabolites with the chemical structure shaped as that of 17β-estradiol and mimic estrogenic actions in mammals[10]. Since they are endowed with both estrogenic and antiestrogenic properties, these natural selective estrogen receptors modulators are now promoted as a preventive alternative against estrogen-dependent cancers[11].

Based on the traditional claims, our previous work focused on *Crateva adansonii* DC (syn: *Crateva réligiosa* auct., family: Capparaceae). This plant is a small tree of 5-6 m high that grows in Sahelian and Sudanese area, including the Far-North region of Cameroon. It is commonly called "varun" or "garlic pear" in English[12]; "migingirgingir" in Giziga and "scramataihi" in Fufulde. An infusion of *C. adansonii* leaves and stem twigs is used to treat constipation and for intimate washing of postmenopausal women[13]. The bark is used to cure uterine affections and inflammatory conditions[12,14]. According to Arbonnier[15], this plant is traditionally used in sub-Saharan Africa to treat cancer, which raised our interest for this plant. The DCM/MeOH extract of *C. adansonii* DC was found to prevent breast tumour chemically induced in Wistar rats, whereas it showed weak cytotoxic effect against MCF-7 cells (CC_{50} = 289 µg/mL). In addition, this extract was found to decrease uterus wet weight, uterine and vaginal epithelial heights in rat undergoing breast cancer investigation[16]. These findings suggest that the aforesaid extract might have estrogenic/antiestrogenic phytoconstituents. The UHPLC-ESI-QTOF-MS analysis, lead to identify citric acid ester derivatives, arylpropanoid, phenylpropanoid, flavane, sesquiterpene derivatives, gallotannin and lignans as the main metabolites of *C. adansonii* DCM/MeOH extract[16].

To the best of our knowledge, there is no published study investigating estrogenic/antiestrogenic activity of *C. adansonii*. To verify this hypothesis, the ability of *Crateva adansonii* DCM/MeOH extract to induce estrogenic and/or antiestrogenic effects in MCF-7–human ER+ breast cancer was tested. *In vivo* testing, a 3-day uterotrophic assay in ovariectomized adult rats [a classical tool for the detection of estrogenicity of chemicals][17], was further used.

Materials and Methods

Chemicals

The 17β-Estradiol benzoate [(Estra-1,3,5(10)-trien-3,16α,17β-triol), purity ≥98%] was obtained from Sigma-Aldrich (Hamburg, Germany). Estradiol valerate (Progynova® 2mg) was purchased from DELPHARM (Lille, France). The 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES, purity ≥99.5%) was purchased from Ludwig Biotecnologia Ltda (Alvorada, RS, Brazil). Trypan blue was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Cultilab (Campinas, SP, Brazil). The cell culture mediums were purchased from Sigma-Aldrich (St. Louis, MO, USA). Serum and antibiotics were purchased from GIBCO (Grand Island, NY). Penicillin (xtapen®) was provided by CSPC Zhongnuo pharmaceutical (Shijiazhuang City, China). Diclofenac (Dicloecnu®) was provided by ECNU pharmaceutical (Yanzhou City, China).

Plant material

The stem barks of *C. adansonii* DC. (Capparaceae) were harvested in Moutourwa (Far-North Region of Cameroon) on the 16th April 2015 (pluvial season) around 11:00 a.m. The plant was localized at the geographical coordinates of 10°21’082’’ North, 014°18’979’’ East and 472±3 m of altitude with a “GARMIN” Global positioning System. The sample used in this study was identified by Dr. Gilbert Todou at the National Herbarium of Cameroon in Yaounde, where the voucher specimens (36359/HNC) were registered and deposited for ready reference.

Preparation of extract

The stem barks of *C. adansonii* were cleaned and dried under shade in an aerated place. The well-dried stem barks of *C. adansonii* were pulverized by electronic grinding. Extract preparation were performed as previously described[16]. Briefly, 2000 g of powder was macerated in 5 L of DCM/MeOH mixture (v/v: 1/1) for 3 days at room temperature (23°C). The process was repeated twice and 33.18 g (1.66% of yield) of crude extract was obtained after filtration through a Whatman N°4 filter paper and evaporation of solvents using a rotary evaporator in vacuum under reduced pressure (from 875 to 337 mbar) at 40°C.

In vitro experiment

Cell line system

The MCF-7 human ER-positive breast adenocarcinoma cells were obtained from the Rio de

Janeiro Cell Bank (Federal University of Rio de Janeiro, Brazil).

Cell culture

MCF-7 cells were cultured in RPMI-1640 medium supplemented with 10% of FBS. Cell culture was also supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10 mM HEPES. The cell culture was maintained at 37°C in a 5% CO_2 humidified atmosphere and pH 7.4. Every two days, cells were passaged by removing 90% of the supernatant and replacing it with fresh medium. Viable cells were checked at the beginning of the experiment by Trypan Blue dye exclusion test.

Cytotoxicity

The cytotoxicity of *C. adansonii* DCM/MeOH extract was evaluated by Alamar Blue (resazurin) assay in MCF-7 cells. This assay evaluates the mitochondrial production as a measurement of cell viability. For this to be done, a density of 1×10^4 cells/well was seeded in a 96-well plate in 100 µL of culture medium. After 24 h to permit their adhesion, cells were exposed for 24 h to the *C. adansonii* extract at concentrations ranging from 10^{-8} to 10^{-5} µg/mL. Experiment was performed in triplicate and repeated three times.

E-screen assay

Estrogenic and antiestrogenic effects of *C. adansonii* DC DCM/MeOH extract were assessed *in vitro* by a simple and sensitive E-screen cell proliferation assay with MCF-7 cells. This assay determines the estrogenicity/antiestrogenicity of compounds indirectly through measurement of the proliferation of MCF-7 cells. For this purpose, the technique adapted from those of Resende *et al.*[18] was used. Briefly, cells were trypsinized and seeded in 24-well plates at an initial concentration of 50000 cells per well in 10% FBS in RPMI. After 24 h of incubation (37°C, 5% CO_2) to permit their adhesion, cells were washed with phosphate-buffered saline (PBS) and the Serum Replacement 2 (0.5×) supplemented phenol red-free RPMI was substituted for the seeding medium. The *C. adansonii* DCM/MeOH extract was added to the experimental medium at concentrations from 10^{-8} to 10^{-5} µg/mL. For antiestrogenicity tests, before incubation, 1×10^{-8} M of 17β-estradiol was added to the wells. Steroid-free experimental medium consisted to negative control while cells treated with 1×10^{-8} M of 17β-estradiol served as positive control. There were also a solvent control (DMSO at 0.01%) and a medium control (10% FBS in RPMI). The assay was stopped after 144 h by removing the medium from wells, and then cells were treated with cold 10% trichloracetic acid and incubated at 4°C for 1 h. Thereafter they were washed four times with tap water and dried. Furthermore, cells were stained during 30 min with 0.057% (w/v) SRB dissolved in 1% acetic acid. Wells were rinsed four times with 1% acetic acid and air dried. Bound dye was solubilized with 10 mM Tris base (pH 10.5) in a shaker. Finally, aliquots were read in a Biotek EL800 absorbance reader (Winoosky, USA) at 510 nm. Results expressing the estrogenic activity were showed as mean ± standard error of mean of the proliferative effect (PE), which represents the maximum proliferation induced by the compounds. This parameter was calculated according to Schiliro´*et al.*[19], and is the ratio between the highest cell number achieved with the sample or 17β-estradiol and the cell number in the solvent control (0.01% DMSO): *PE = max cell number of sample/cell number of DMSO control*. The estrogenic activity of a sample was determined as the relative proliferative effect (RPE%). The RPE compares the maximum proliferation induced by a sample with that induced by 17β-estradiol: *RPE% = [PE for sample/PE for 17β-estradiol] ×100*.

***In vivo* experiment**

Animals

Forty five adult female Wistar rats, aged 60-70 days at the start of experiment and weighing around 150-170 g, were supplied by the breeding facility of the Laboratory of Animal Physiology, University of Yaounde I (Yaounde, Cameroon). Animals were housed in plastic cages in groups of five at room temperature. The animals had free access to drinking water and to a standard pellet rat diet. The composition of animal diet was: corn (36.7%), bone flour (14.5%), wheat (36.6%), fish flour (4.8%), crushed palm kernel (7.3%), sodium chloride (0.3%) and vitamin complex (Olivitazol® - 0.01%). Rats were treated in accordance with the guidelines and procedures of animal bioethics of the Cameroon National Institutional Ethic Committee (CEE Council 86/609), which adopted all procedures recommended by the European Union on the protection of animals used for scientific purposes.

The 3-day uterotrophic assay

Forty five female Wistar rats were pretreated with a single intramuscular dose of long acting penicillin and diclofenac (10 mg/kg and 3 mg/kg, respectively) the day before ovariectomy. Further, animals were bilaterally ovariectomized (OVX) using the dorsal approach under Diazepam and ketamin anesthesia (respectively 10 mg/kg and 50 mg/kg body wt.; i.p.). After 14 days of endogenous hormonal decline[15], animals were randomly distributed into 9 groups of five animals each (n = 5). The negative control group (Control) was treated with the vehicle (2% ethanol). The positive control group received estradiol valerate (E2V) as standard drug at the optimal dose of 1 mg/kg body wt./day. One group was co-treated with E2V (1 mg/kg body wt.) and the pure anti-estrogen faslodex (ICI 182,780) at the dose of 300 µg/kg body wt./day. The remaining groups received DCM/MeOH extract of *C. adansonii* DC at doses of 75, 150 and 300 mg/kg body wt./day for 3 days in presence or not of E2V (for antiestrogenic evaluation). All treatments were administered by oral route (2 mL/150 g) for 3 days. Twenty-four hours past last administration, animals were euthanized by decapitation. The uterine wet weight, total protein levels in uterus, uterine and vaginal epithelial heights and mammary gland were assessed as described before by Zingue *et al.*[16].

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 softwares, all provided by Zeiss (Hallbermoos, Germany), the histomorphology of the mammary glands, as well as the uterine and vaginal epithelial heights, were assessed from 5-µm 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.*[20].

Statistical analysis

Results are presented as means ± standard error of mean (SEM). *In vitro* experiment was performed in triplicates and repeated three times. All formulas and functions were calculated with Microsoft Excel software. Data analysis was performed with GraphPad Prism 5.0 software, using the ANOVA test followed by the Dunnett's post hoc test. Differences were considered significant at a probability level of 5% (P <0.05).

Results

***In vitro* experiments**

Cytotoxicity

C. adansonii DCM/MeOH extract did not induced cytotoxic effects in MCF-7 cells at concentrations range between 10^{-8} and 10^{-5} µg/mL (Fig. 1A).

E-screen assay

Figure 1B depicts effects of *C. adansonii* extract in MCF-7 cells proliferation. It can be observed that E2B induced a significant (P <0.001) increased of MCF-7 cells yield. *C. adansonii* extract did not induce MCF-7 cells proliferation as compared to DMSO control. However, a significant and concentration-dependant inhibition of cells proliferation, meaning antiestrogenic effect was noted with *C. adansonii* extract when co-treated with E2B.

***In vivo* experiments**

Effects on uterus

A 3-day treatment with E2V (1 mg/kg) significantly (P <0.001) increased uterine wet weight, uterine epithelium height as well as uterine total protein level as compared to the negative control group (OVX) (Fig. 2). The co-treatment of faslodex with estradiol (E2V) significantly reduced total

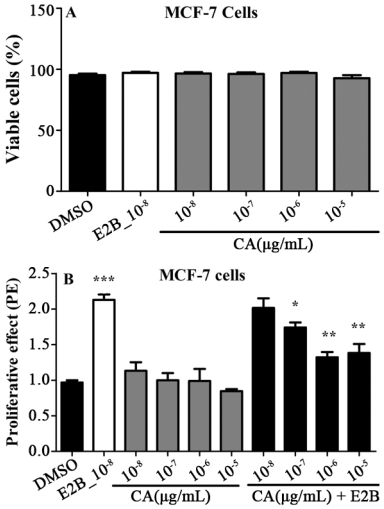

Fig. 1 — Effects of *Crateva adansonii* extract on MCF-7 cells proliferation. Its effect was investigated by measuring E-screen assay. [The relative MCF-7 cells yields (PE) were measured in the presence of DMSO (0.01%), 17β-estradiol (E2B, 10 nM) and CA = *C. adansonni* DCM/MeOH extract. PE = max cell number of sample/cell number of DMSO control. * P <0.05, *** P <0.001 as compared to the DMSO control]

protein level (P <0.01), uterine wet weight (P <0.001) and uterine epithelium height (P <0.001) as compared to the positive control group (E2V). *C. adansonii* DCM/MeOH extract did not induce any changes in uterine parameters at all tested doses compared to OVX control group. However, when combined with E2V, it was observed a significant (P <0.05) reduction of uterine wet weight at doses of 75 and 150 mg/kg, total protein level in uterus at doses of 75 and 300 mg/kg; while only the dose of 75 mg/kg significantly (P <0.01) reduced uterine epithelium height as compared to E2V group (Fig. 2).

Effects on vagina

Fig. 3 depicts the microphotographs of vagina obtained after a 3-day treatment with *C. adansonii* DCM/MeOH extract. As expected, E2V treatment induced a significant increase (P <0.001) of vaginal epithelium height as compared to OVX group. *C. adansonii* extract failed to alter vaginal epithelium height at all tested doses. However, when combined with the E2V, it was noted a significant (P <0.01) reduction of this parameter at the dose of 75 mg/kg.

Effects on mammary glands

Fig. 4 shows microphotographs of mammary glands obtained after a 3-day treatment with *C. adansonii* DCM/MeOH extract. E2V treatment increased the size of the acini as well as epithelial cells of the acini, and there is also an abundant eosinophile secretions inside the acini. Faslodex

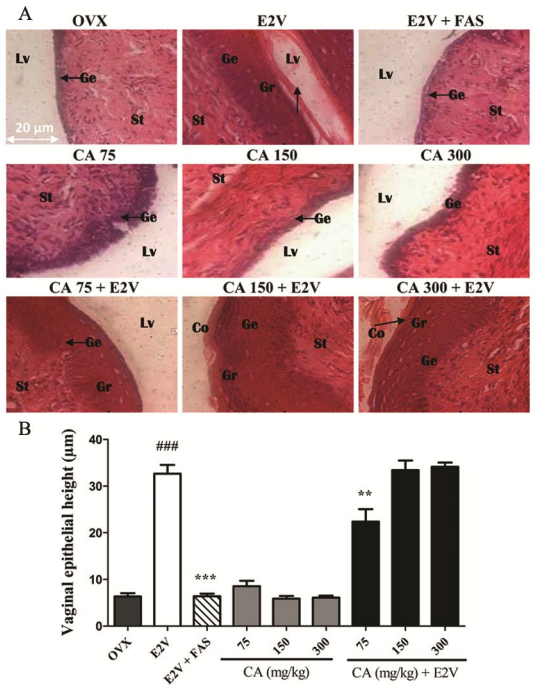

Fig. 3 — Effects of a 3 day treatment with *C. adansonni* DCM/MeOH extract on the vaginal epithelium: (A) micro-photographs; and (B) epithelial height. [OVX = OVX animals treated with the vehicle; E2V = OVX animals treated with estradiol valerate at 1 mg/kg body wt.; E2V + FAS = OVX animals co-treated with E2V at 1 mg/kg body wt. and faslodex (300 µg/kg); CA = OVX animals treated with *C. adansonni* DCM/MeOH extract at doses of 50, 150 and 300 mg/kg body wt.; and CA + E2V = OVX animals co-treated with *C. adansonni* DCM/MeOH extract at doses of 50, 150 and 300 mg/kg body wt. and E2V (1 mg/kg). Lv = vaginal lumen; Co = stratum corneum; Gr = stratum granulosum; Ge = stratum germinativum; St= Stroma]

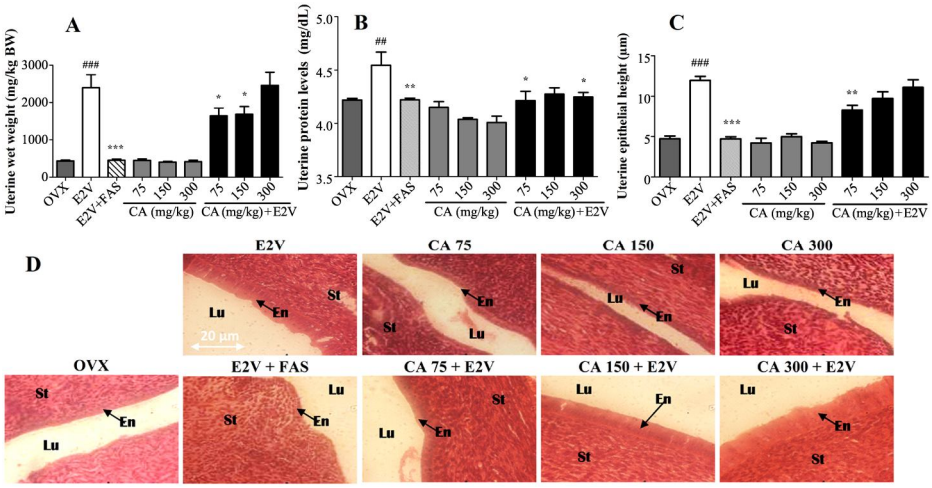

Fig. 2 — Effects of a 3-day treatment with *C. adansonni* DCM/MeOH extract on (A) the uterine wet weight; (B) total protein levels in uterine; (C) uterine epithelial height; (D) and microphotographs. [OVX = OVX animals treated with the vehicle; E2V = OVX animals treated with estradiol valerate at 1 mg/kg body wt.; E2V + FAS = OVX animals co-treated with E2V at 1 mg/kg body wt. and faslodex (300 µg/kg); CA = OVX animals treated with *C. adansonni* DCM/MeOH extract at doses of 50, 150 and 300 mg/kg body wt.; and CA + E2V = OVX animals co-treated with *C. adansonni* DCM/MeOH extract at doses of 50, 150 and 300 mg/kg body wt. and E2V (1 mg/kg). **P* <0.05, ***P* <0.01 as compared with control. Lu = uterine lumen; En = Endometrium; St = Stroma]

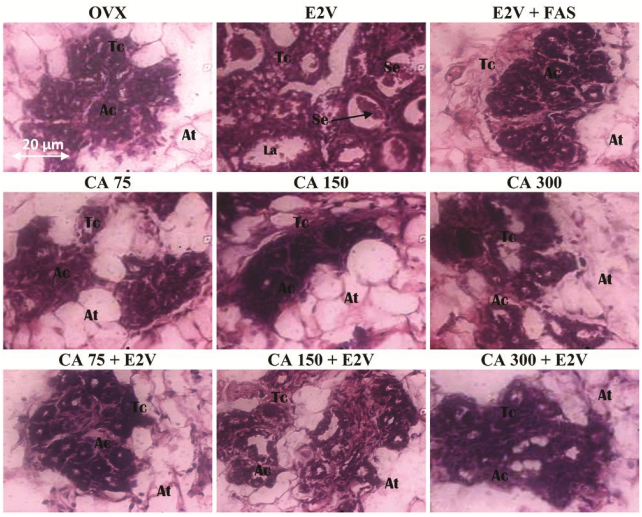

Fig. 4 — Effects of a 3-day treatment with *C. adansonni* DCM/MeOH extract on mammary gland. [OVX = OVX animals treated with the vehicle; E2V = OVX animals treated with estradiol valerate at 1 mg/kg body wt.; E2V + FAS = OVX animals co-treated with E2V at 1 mg/kg body wt. and faslodex (300 µg/kg); CA = OVX animals treated with *C. adansonni* DCM/MeOH extract at doses of 50, 150 and 300 mg/kg body wt.; and CA + E2V = OVX animals co-treated with *C. adansonni* DCM/MeOH extract at doses of 50, 150 and 300 mg/kg body wt. and E2V (1 mg/kg). La = lumen of alveoli; Ep = aveoli epitheluim; At = adiposite tissue; Se = eosinophil secretion]

inhibited estrogenic effect by reducing the acini height, as well as the eosinophile secretions in acini. No estrogenic-like effect was noted with *C. adansonii* treatment in this parameter. However, it counteracted the effects of E2V in mammary glands by reducing the acini heights and eosinophile secretion in acini when co-administered with E2V.

Discussion

In certain regions of the world, particularly in Asia, a low incidence of breast cancer has been observed. These women have diets that are high in soy and low in fat[21]. These soy beneficial effects have been attributed to phytoestrogens, which are plant derivatives that bear a structural similarity to 17-β estradiol and act in a similar manner. In our previous work, *Crateva adansonii* extract prevented breast tumours induced in rat and significantly reduced the uterine wet weight as compared to both normal and tumour groups at the dose 75 mg/kg[16]. In order to contribute to a better underlying mechanism of this plant constituents, its estrogenic/antiestrogenic effects were assessed. *C. adansonii* extract counteracted MCF-7 cells proliferation induced by E2V using E-creen assay. This assay, initially used by Soto *et al.*[22] compares cell proliferation after estradiol treatment that yield by tested substances. Resende *et al.*[18] reported that a relative proliferative effect (RPE) ≥80 corresponds to estrogenic activity and suggest that the compound may have agonistic activity through ERα. No MCF-7 cells proliferation was observed with *C. adansonii*, while a concentration-dependent decrease of the E2-induced cell proliferation was observed when this extract was co-administered with E2 as compared to E2 treatment only. This result suggests that *C. adansonii* might contain compounds that may enter in competition with ERs and collect the corepressors responsible for antiestrogenic effects[23]. *In vivo C. adansonii* extract failed to induce estrogenic effect, however it counteracted E2-induced increased in uterine wet weight, uterine and vaginal epithelial height. These results suggest that *C. adansonii* extract could have secondary metabolites with antiestrogenic properties, which in turn can account for its antitumour effects. Antiestrogenic effects are a needed effect on estrogen-dependent breast cancer, which are approximately two thirds of all breast cancers. *C. adasonnii* extract exerts beneficial breast cancer preventive action by competing with endogenous estrogen, thus limiting the mammary gland ductal and lobular proliferation. Flavane and flavonol (quercetin) detected in this extract in our previous report[16], are well known as phytoestrogens. Just like SERMS, phytoestrogens can bind to either ERα or ERβ however; phytoestrogens appear to have a higher affinity for ERβ[24]. This affinity may be dose-dependent but overall phytoestrogens have a significantly lower affinity for the ER than estradiol[25]. Indeed, it has been found that at a concentration 100 to 1000 times higher than estradiol, phytoestrogens enter in competition with endogenous estrogens for ERs by preventing estrogens stimulated growth in mammals[26]. Since MCF-7 cells can elicit estrogenic/antiestrogenic responses involving both genomic and non-genomic pathways[19]; it can be hypothesized that phytoestrogens detected in *C. adansonii* DCM/MeOH extract might modulate endogenous estrogens concentration by linking or inactivating some enzymes which can influence bioavailability of sex hormone binding globulin and/or enter in competition with E2 for ERs[27].

Conclusion

C. adansonii extract is now reported to endow antiestrogenic effects. This might account to its antitumour activity. Flavonoids detected in this extract may be the key compounds that exert this activity. In depth investigations are needed to isolate and assess the affinity as well as the ability of these compounds to transactivate the estrogen receptors.

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