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Cytogenotoxic and antihyperplastic effects of the aqueous extracts of *Cymbopogon citratus* (Poaceae) and *Citrus medica* (Rutaceae)

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Keywords

Allium cepa assay; *Citrus medica*; *Cymbopogon citratus*; Cytogenotoxicity; Uterine hyperplasia; Rats

Historic

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Abstract

Cymbopogon citratus and *Citrus medica* are commonly consumed in combination as an herbal tea to prevent or treat various conditions including cancers. This study evaluated the cytogenotoxic and antihyperplastic effects of aqueous extracts of *C. citratus* (AECC) and *C. medica* (AECM). Both extracts were chemically screened for the quantification of phytochemicals with genotoxic properties, and their antioxidant activities were evaluated *in vitro*. The cytogenotoxicity was evaluated on *Allium cepa* exposed to increasing concentrations (0.25, 0.5, and 1 mg/mL) of AECC and AECM and to mixtures of the two extracts [Mix 1 (AECC 0.25 + AECM 0.25), Mix 2 (AECC 0.5 + AECM 0.5) and Mix 3 (AECC 1 + AECM 1)] for 96 h. They were then examined macroscopically and microscopically for evaluation of mitotic index and chromosomal aberrations. The antihyperplastic effects of the two extracts were evaluated on the endometrium of ovariectomized rats. Endometrial hyperplasia was induced with tamoxifen (10 mg/kg; *ip*). Plant extracts were administrated orally for 37 days at the doses of 8, 40 and 200 mg/kg BW for AECC; 34, 170 and 850 mg/kg BW for AECM. The co-administration of the two extracts was carried out according to the following scheme: Mix 1: AECC 8 + AECM 34; Mix 2: AECC 40 + AECM 170; and Mix 3: AECC 200 + AECM 850. Animals were then sacrificed for biological analyses. Results showed that the contents of phenols, flavonoids, and tannins were lower in AECM than in AECC. AECC was also found to have greater antioxidant capacity than AECM. Both extracts induced chromosomal aberrations and reduced mitotic index ($p < 0.001$) in meristematic cells of *A. cepa*. These extracts also inhibited the hyperplastic effect of tamoxifen by reducing the uterine epithelium to a single cell layer ($p < 0.001$). This effect was associated with decreased serum estradiol levels, uterine oxidative stress and increased cytotoxicity. In conclusion, *C. citratus* and *C. medica* are endowed with genotoxic, mitodepressant, and antihyperplastic properties, justifying at least in part their use against cancers. The joint use of the two medicinal plants has proven to be more effective than their separate use.

1. Introduction

Cancer is a proliferative disease recognized as one of the greatest health problems in the world [1]. Despite their diversity, cancers have the following common characteristics: continuous and sustained cell proliferation, insensitivity to growth suppressors, resistance to apoptosis, induction of angiogenesis, and activation of invasion and metastasis [2]. Cell proliferation is defined as an increase in the number of cells resulting from cell growth and division [3]. Disordered cell proliferation leads to tissue hyperplasia and later cancer if left untreated [4,5]. Nowadays, enormous efforts are being made to develop complementary or alternative anticancer treatments to allopathic therapies which have proven to be expensive and associated with significant side effects, resistance, and recurrence [6].

Plants with cytogenotoxic and antiproliferative abilities are considered potential anticancer agents [7]. *Cymbopogon citratus* (Poaceae) *and Citrus medica* (Rutaceae) are plants of the Cameroonian pharmacopeia commonly consumed in combination as herbal tea to prevent or treat various ailments including cancers [8]. Previous studies have shown that *C. citratus* is endowed with anti-inflammatory and antioxidant properties, cytotoxic and genotoxic activities [9,10,11]. *Citrus medica* can induce apoptosis and inhibit cell proliferation [12,13]. However, no study has so far attempted to provide scientific proof of the anticancer potential of these Cameroonian medicinal plants. The present study therefore aimed to evaluate the cytogenotoxic and antihyperplastic effects of aqueous extracts of *C. citratus* (AECC) and *C. medica* (AECM).

The cytogenotoxic effects of AECC and AECM were evaluated on actively proliferating cells in onion bulbs (*Allium cepa*: Alliaceae). *A. cepa* bioassay is commonly used as a short-term and cost-effective bioindicator for the evaluation of cytogenotoxicity of chemicals, as it has rapid root growth rate (indicator of rapid cell multiplication) and reduced number of large chromosomes (2n = 16 chromosomes) [14,15]. This bioassay is used to assess cytotoxic and genotoxic endpoints such as chromosomal aberrations, nuclear alterations, root growth inhibition and mitotic index alterations [13]. Mitotic Index (MI) is defined as the ratio between the number of cells in a population undergoing mitosis and the total number of cells in a population [10]. The aberration index (AI) is the ratio of the number of cells in a population with chromosomal aberrations to the total number of cells in a population [10]. Several aberrations can be observed like chromosomal adherence, chromosomal break, chromosomal bridge, chromosomal laggard, disturbed anaphase, and disorganized metaphase. To confirm

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the antiproliferative capacity of both plants in animal organisms, their effects were further evaluated on tamoxifen-induced endometrial hyperplasia in ovariectomized rats. Indeed, endometrial hyperplasia is a disordered proliferation of endometrial glands resulting from chronic exposure to unopposed stimulation of endometrial tissue by estrogens [5]. It is the precursor lesion of endometrial adenocarcinoma, a common malignancy of the female reproductive tract [16,4,5]. The radical scavenging potentials of the two extracts were also investigated.

2. Materials and methods

2.1. Plant material: Collection and extractions

The onion bulbs were obtained from the Institute of Agricultural Research for Development (IARD) of Maroua (Far North Region, Cameroon) and compared to the specimen recorded in the National Herbarium of Cameroon under the number Bot.0017/1973/0002. The fruits of *C. medica* were harvested in Melong (Littoral Region, Cameroon), *C. citratus* was collected in Tobe-Bandjoun (West Region, Cameroon) and both plants were compared to the samples registered at the national herbarium of Cameroon under the numbers 65106HNC and 18628RF/Cam, respectively.

The preparation of the aqueous extract of *C. citratus* consisted of boiling 300 g of fresh and clean samples of *C. citratus* in 3 L of distilled water for 30 min. After cooling, the decoction obtained was filtered and lyophilized to obtain 27.3 g (yield: 9.1%) of the crude aqueous extract of *C. citratus* (AECC). For the preparation of the aqueous extract of *C. medica* fruits (lemons), 50 fresh and clean lemons (1 kg) were cut and infused in 3 L of boiling distilled water for 30 min. The infusion was filtered and lyophilized to obtain 54.8 g (yield of 5.48%) of the crude aqueous extract of *C. medica* (AECM).

2.2. Quantitative chemical analysis

Total phenol, tannin, and flavonoid contents in AECC and AECM were determined by the method described by Ramde-Tiendrebeogo et al.[17], the modified Folin-Ciocalteu method and the aluminum chloride colorimetric method, respectively [18]. Briefly, the quantification of total phenol content in plant extracts was realized by mixing 200 µl of each extract with 200 µl of 2N Folin-Ciocalteu reagent, and 400 µl of 20% sodium carbonate solution. The mixtures were stirred and incubated in a water bath at 40°C for 20 minutes. The experiment was carried out in triplicate. The absorbance was read at 760 nm. Total phenolic content was expressed as milligram gallic acid equivalent/gram of extract (mg GAE/gE), using the equation obtained from the calibration curve of gallic acid ($y=1963x$; $R^2 = 0.9926$).

The total tannin content in both extracts was determined by the modified Folin-Ciocalteu method. The reaction mixture consisted of 200 µl of each extract, 200 µl of 2N Folin-Ciocalteu reagent, and 400 µl of 20% sodium carbonate solution. The mixtures were stirred and incubated in a water bath at 40°C for 20 minutes. The experiment was carried out in triplicate. The absorbance was read at 700 nm. Total tannin content was expressed as milligram tannic acid equivalent/gram of extract (mg TAE/gE), using the equation obtained from the calibration curve of tannic acid ($y=0.0005x + 0.017$; $R^2 = 0.9873$).

The total flavonoid content was determined using the aluminum chloride colorimetric method [18]. In brief, 1500 µl of distilled water and 30 µl of sodium nitrite at 5% were added to 100 µl of each extract. After 5 min of incubation at room temperature, 30 µl of aluminum chloride (10%) and 200 µl of sodium hydroxide (1 M) were added to the mixtures. The experiment was carried out in triplicate. The absorbance was measured at 510 nm. Total flavonoid content was calculated as milligram quercetin equivalent/gram of extract (mg QE/gE), using the equation obtained from the calibration curve of quercetin ($y=300.75x+0.0897$; $R^2 = 0.9956$).

2.3. In vitro antioxidant activity

The antioxidant potential of AECC and AECM was estimated *via* their ability to scavenge free radicals using 2.2-diphenyl-1-picryl hydroxyl (DPPH) and Ferric-reducing power assay (FRAP) tests.

2.3.1. DPPH test: The DPPH test of aqueous extracts of *C. citratus* and *C. medica* was performed following the method described by Mensor et al.[19]. Briefly, in each well of a 96-well plate, 20 µL of distilled water was introduced into the last seven rows. This was followed by the introduction of 20 µL of the aqueous solutions of the aqueous extracts of *C. citratus* and *C. medica* (2 mg/mL) into the first two wells of each column (4 columns were used for one sample) and successive serial dilutions of factor 2 were carried out in the other wells while maintaining the volume at 20 µL. A volume of 180 µL of aqueous DPPH solution (0.08 mg/mL) was again introduced into each of the wells of the first three columns, while a volume of 180 µL of distilled water was introduced into each of the wells of the fourth column. Plates containing 200 µL of final solution per well were incubated for 30 min in the dark and at room temperature. At the end of the 30 min incubation, the optical densities were read on a spectrophotometer (FLUOstar Omega microplate reader) at 517 nm and converted into percentages of antioxidant activity. Vitamin C (L-ascorbic acid) (2 mg/mL) was used as a positive control. For each sample, three replicates were performed. The percentages of antioxidant activity of each sample were calculated as follows: Antioxidant activity (%) =100*[AD - (AE – AB)] / AD

AD: Absorbance of DPPH; AE: Absorbance of extract; AB: Absorbance of blank

The different percentages of antioxidant activity of each extract were used to determine the inhibitory concentration value (IC_{50}) (the concentration of the sample that is required to scavenge 50% of the DPPH free radical) [20]. To do this, the regression lines were drawn from the values of the different percentages of antioxidant activity of DPPH and the decimal logarithm of the concentrations of the samples [% of antioxidant activity = f (logC)]. The equations of the regression lines ($y = ax + b$) were used. Assuming each time $y = 50$, we get $IC_{50} = 10^x$ where $x = (50 – b)/a$ (µg/mL).

2.3.2. FRAP assay: The reducing power of the aqueous extracts of *C. citratus* and of *C. medica* was determined according to the protocol described by Benzie et al.[21]. To do this, a volume of 5 µL of each extract (2 mg/mL) was mixed with 95 µL of FRAP reagent. The mixture was incubated for 30 min at 37°C in the dark. After incubation, the optical density was read on a spectrophotometer (FLUOstar Omega microplate reader) at 593 nm. Vitamin C (2 mg/mL) was used as the positive control. The antioxidant power of the sample was calculated from the calibration curve of the FeSO4 solution (the number of moles of the FeSO4 solution varying from 156.25 µmol to 10,000 µmol).

2.4. In vivo tests

2.4.1. Experimental animals

Female Wistar rats aged 8 to 10 weeks were provided by the Research Unit of Animal Physiology and Phytopharmacology of the University of Dschang (Cameroon). They were fed a standard soy-free diet and were having free access to drinking water and diet *ad libitum*.

2.4.2. Allium cepa bioassay

The relatively same-sized onion bulbs obtained from IARD of Maroua (Far North Region, Cameroon) were used. The scaly outer leaves of the onion bulbs were carefully removed by hand while the dried roots were

shaved off with a sharp razor blade to expose fresh meristematic tissues. The bulbs were then placed in freshly obtained distilled water for 24 hours, after which the normally growing bulbs were selected and divided into 10 treatment groups as follows:

- Control (n = 10): bulbs grown in distilled water;
- [AECC]: bulbs grown in the aqueous extract of *C. citratus* at the concentrations of 0.25 mg/ml (AECC 0.25; n = 10), 0.5 mg/ml (AECC 0.5; n = 10) and 1 mg/ml (AECC 1; n = 10);
- [AECM]: bulbs grown in the aqueous extract of *C. medica* at the concentrations of 0.25 mg/ml (AECM 0.25; n=10), 0.5 mg/ml (AECM 0.5; n = 10) and 1 mg/ml (AECM 1; n = 10);
- [Mix]: bulbs grown in the mixture of aqueous extracts of *C. citratus* and *C. medica* at the lowest [Mix 1 (AECC 0.25 + AECM 0.25); n = 10], medium [Mix 2 (AECC 0.5 + AECM 0.5); n = 10] and highest [Mix 3 (AECC 1 + AECM 1); n = 10] concentrations.

After 4 days of treatment, the macroscopic evaluation (root number and length) and microscopic analysis [mitotic index (MI) and aberration index (AI)] of *A. cepa* roots were subsequently done. Photomicrographs of the root tip cells were taken using an Olympus model microscope with a 5 megapixel digital microscopic eyepiece camera. The standard procedures described by Erhabor and Erhabor.[10] were adopted. The following parameters were measured:

- Growth rate: GR (%) = 100 x (length of treated roots / length of control roots)
- Mitotic index: MI (%) = 100 x (Number of dividing cells / Number of examined cells)
- Phase index: PI (%) = 100 x (Number of cells in P, M, A or T / Number of examined cells); with P: prophase; M: metaphase; A: anaphase; T: telophase
- Aberrations Index: AI (%) = 100 x (Number of aberrant cells / Number of examined cells)

2.5. Experimental protocol for endometrial hyperplasia

The ability of extracts to inhibit the hyperplasic effect of tamoxifen on the uterine endometrium of ovariectomized rats was evaluated following the method described by Mvondo et al.[7]. Briefly, 66 rats were ovariectomized (OVX) while 6 rats were sham-operated (SHAM). On the 3rd day post-ovariectomy, all OVX rats received tamoxifen (10 mg/kg) intraperitoneally (*ip*) while sham-operated rats received the vehicle (NaCl 0.9%) through the same route. From the 5th day post-ovariectomy to the end of the experimentation, rats were assigned to the following treatment groups (n=6):

- [SHAM]: sham-operated animals receiving NaCl *ip* and *per os*;
- [OVX]: OVX rats receiving NaCl *ip* and *per os*;
- [TAM]: OVX rats receiving tamoxifen *ip* and NaCl *per os*;
- [AECC]: OVX rats receiving tamoxifen *ip* and AECC (8, 40, and 200 mg/kg) *per os;*
- [AECM]: OVX rats receiving tamoxifen *ip* and AECM (34, 170, and 850 mg/kg) *per os*;
- [Mix]: OVX rats receiving tamoxifen *ip* and AECC and AECM at the lowest (8 and 34 mg/kg, respectively; MixI), medium (40 and 170 mg/kg, respectively; Mix2) and highest (200 and 850 mg/kg, respectively; Mix3) doses, *per os*.

The intraperitoneal administration of tamoxifen or NaCl started third-day post-ovariectomy until the end of the study. The oral administration of extracts started from the 5th-day post-ovariectomy until the end of the study. Co-treatments were performed for 37 consecutive days as Mvondo et al.[7] described. Animals were sacrificed thereafter under anesthesia (10 mg/kg diazepam and 50 mg/kg ketamine) and after a 12 h overnight nonhydric fasting. Blood collected by abdominal artery catheterization was centrifuged at 3000 rpm for 15 minutes at room temperature and the resulting serum was used for the determination of estradiol levels. The uterus of each animal was removed, freed of fatty tissue, and then weighed. The left uterine horn was used for the preparation of homogenates in 0.9% NaCl (0.1 g per 1 ml) while the right uterine horn was fixed in 10% formalin for histological analysis.

2.6. Biochemical analyses

2.6.1. Evaluation of serum estradiol levels

Serum estradiol levels were immunologically determined by enzyme-linked immunorbent assay (ELISA) using a reagent kit (Mouse / Rat Estradiol ELISA) purchased from Calbiotech (El Cajon, California, USA). The absorbance of calibrators and specimen was determined using an ELISA microplate reader, the Multiskan ascent plate reader, purchased from MTX Lab Systems, Inc. (Brandenton, USA). The concentration was evaluated by means of a calibration curve established from the calibrators supplied with the kit.

2.6.2. Assessment of uterine oxidative status

Uterine oxidative status was assessed by measuring tissue levels of malondialdehyde (indicator of oxidative stress), glutathione and superoxide dismutase (antioxidant agents).

Malondialdehyde (MDA) levels were determined by the method described by Gawade et al. [22] which is based on the reaction with the thiobarbituric acid (TBA) at 90°C–100°C. In the TBA test, MDA reacts with the production of a pink pigment having an absorption maximum at 532 nm. The level of malondialdehyde was determined as followed: [MDA] =ΔDO/ε.l.m. where [MDA]: concentration of malondialdehyde (nM/mg of tissue), ΔDO: absorbance of the sample - absorbance of the reagent blank, ε: molar extinction coefficient ($1.56.10^{-4} nM^{-1}.cm^{-1}$), l: length of path (1 cm) and m: mass of tissue collected for the homogenization (mg).

Glutathione (GSH) levels were determined by the method of Sehirli et al.[23]. Indeed, GSH is oxidized by 5,5′-dithiobis-2-nitrobenzoic acid to 5-thio-2-nitrobenzoic acid. The optical density of this yellow colored compound was read at 412 nm. Tissue levels of GSH were determined using the following formula: [GSH] =ΔDO/ε.l.m. where [GSH]: concentration of GSH (nM/mg of tissue), ΔDO: absorbance of the sample - absorbance of the reagent blank, ε: molar extinction coefficient ($1.36.10^{-5} nM^{-1}.cm^{-1}$), l: path length (1 cm), and m: mass of the tissue collected for homogenization (mg).

The superoxide dismutase (SOD) assay was performed according to the methods described by Misra and Fridovich.[24] and Dimo et al.[25]. SOD assay is based on the ability of this enzyme to inhibit the auto-oxidation of adrenalin to adrenochrome in a basic medium. At the end of this test, the absorbance of the product (adrenochrome) obtained after initiating the reaction was evaluated after 30 and 90 seconds at 480 nm and the inhibition percentage (I) of the SOD was determined as follows:

$$\%I = 100 - (DO\ sample/DO\ Blank) * 100$$

One unit of SOD was equivalent to 50% inhibition and the determination of the SOD activity was performed using the following formula: A = 1/(50 x Total proteins)

Total protein levels were determined using the Randox reagent kit following the supplier's recommendations. Briefly, 500 µl of the reagent was added to 10 µl of sample then homogenized and incubated for 10 min at room temperature. The absorbance (A) was measured at 510 nm. Concentrations of total proteins were calculated as follows:

Total Proteins (g/l) = (A sample/A standard) * Standard (g/l)

2.6.3. Histological analysis

Histological analysis of the uterus was assessed to determine whether or not there is hyperplasia of the uterine epithelium. Therefore, 5 µm sections of paraffin-embedded tissues were stained with hematoxylin and eosin. Histological changes were assessed on microphotographs using an Olympus (Tokyo, Japan) microscope equipped with a camera connected to a computer where the image was transferred and analyzed with the Image J1.3 software.

2.7. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) and analyzed by one-way ANOVA followed by the Turkey post-test, using the GraphPad Prism 5.03 software. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Chemical composition of extracts

AECC was found to have higher amounts of phenols (16.20 ± 0.13 mgGAE/gE), flavonoids (5.50 ± 0.21 mQE/gE) and tannins (9.60 ± 0.23 mgTAE/gE), relative to AECM where the quantities of these classes of secondary metabolites were 9.30 ± 0.13 mgGAE/gE, 1.40 ± 0.33 mgQE/gE, and 7.30 ± 0.05 mgTAE/gE, respectively.

3.2. In vitro antioxidant activity

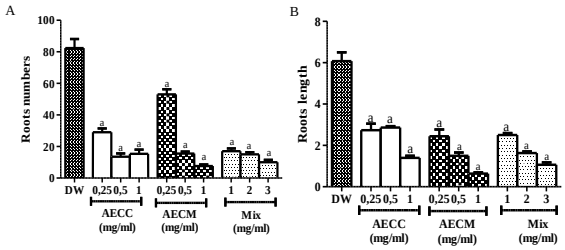
The concentration of vitamin C capable of trapping 50% of DPPH was 8.29 ± 0.13 µg/mL. The concentration of AECC capable of trapping 50% of DPPH proved to be 10 times higher (83.75 ± 0.33 µg/mL) than that of vitamin C. For AECM, this parameter was even higher (134.89 ± 0.65 µg/mL; 16-fold increase) compared to vitamin C.

The results of the FRAP test show that the quantity of Fe^{3+} reduced to Fe^{2+} by AECC was lower by 78% (34.23 ± 0.64 mmol $FeSO_4$/g of extract) than that of vitamin C (160.42 ± 0.55 mmol $FeSO_4$/g vitamin C). The ferric-reducing antioxidant power of AECM was even lower (16.52 ± 0.27 mmol $FeSO_4$/g of extract; 90% decrease) compared to vitamin C.

3.3. Effects of aqueous extracts of *C. citratus* and *C. medica* on the numerical and longitudinal growth of the roots of *A. cepa*

AECC decreased ($p < 0.001$) the number of roots by 64.80%, 83.61%, and 81.31% at the three concentrations tested, respectively, compared to the control. Similarly, AECM reduced ($p < 0.001$) this parameter by 35.64%, 81.18%, and 90.77% at the three concentrations tested, respectively. The mixture of the two extracts further reduced ($p < 0.001$) this parameter by 79.36%, 81.79%, and 87.86% in the three test groups, respectively (Figure 1A).

AECC reduced ($p < 0.001$) the average length of the roots by 55.09%, 52.96%, and 76.97% at the three concentrations tested, respectively, compared to the control. AECM induced a similar effect as it decreased ($p < 0.001$) root length by 59.86%, 75.32%, and 89.47% at the three concentrations tested, respectively. The mixture of the two extracts reduced ($p < 0.001$) this parameter by 58.88% ($p < 0.05$; Mix 1), 73.13% ($p < 0.001$; Mix 2) and 82.56% ($p < 0.001$; Mix 3) (Figure 1B).



AECC: aqueous extract of *C. citratus*; AECM: aqueous extract of *C. medica*; Mix: AECC + AECM. The results are expressed as mean ± standard error of mean (S.E.M.), n = 10; [a] $p < 0.001$ compared to distilled water.

Figure 1: Effects of treatments on the number (A) and length (B) of *Allium cepa* roots.

3.4. Effects of extracts on mitosis

AECC significantly ($p < 0.001$) decreased the prophase index (PI) by 70.04%, 74.46%, and 82.32% at the tested concentrations, respectively, compared to the control. AECM further reduced ($p < 0.001$) PI by 72.99%, 79.70%, and 84.28% at the different concentrations tested, respectively. Similarly, the mixture of the two extracts reduced ($p < 0.001$) PI by 87.56% (Mix 1), 88.54% (Mix 2) and 90.34% (Mix 3) (Table I).

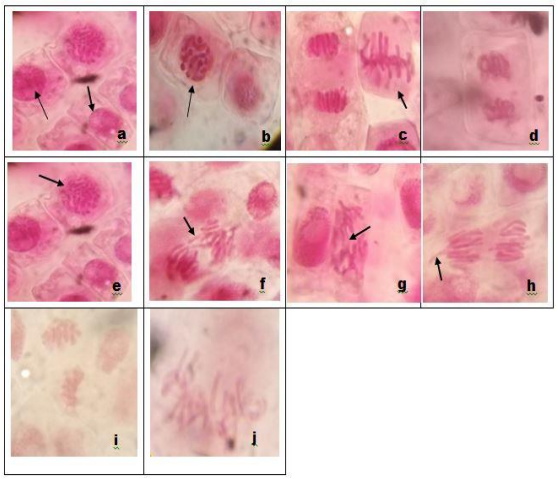
The mitotic index (MI) therefore decreased ($p < 0.001$) by 67.84%, 73.12% and 81.12%, after exposure to AECC at the three concentrations tested, respectively, compared to the control. Similarly, AECM decreased ($p < 0.001$) MI by 70.56%, 78.24%, and 83.34% at the three concentrations tested, respectively. The mixture of the two extracts further reduced ($p < 0.001$) the MI by 85.60%, 87.68%, and 89.28% in the three test groups, respectively (Table I).

Table I: Effects of *C. citratus* and *C. medica* on mitosis phases, mitotic index and chromosomal aberrations

Groups	Number of dividing cells	Phases of mitosis (%)				Mitotic index (MI) (%)
		PI	mI	AI	TI	
Control	125±5.62	12.22±0.43	0.16±0.05	0.08±0.03	0.04±0.02	12.50±0.55
C. citratus						
AECC 0.25	40.20±2.90 [a]	3.66±0.19 [a]	0.18±0.03	0.12±0.03	0.06±0.02	4.02±0.28 [a]
AECC 0.5	33.60±2.90 [a]	3.12±0.20 [a]	0.18±0.03	0.04±0.02	0.02±0.02	3.36±0.28 [a]
AECC 1	23.60±4.65 [a]	2.16±0.35 [a]	0.10±0.04	0.04±0.02	0.06±0.04	2.36±0.46 [a]
C. medica						
AECM 0.25	36.80±3.30	3.30±1.70 [a]	0.16±0.05	0.14±0.05	0.08±0.05	3.68±0.32 [a]
AECM 0.5	27.20±3.28	2.48±0.22 [a]	0.12±0.03	0.08±0.03	0.04±0.02	2.72±1.93 [a]
AECM 1	20.20±1.81	1.92±0.10 [a]	0.06±0.04	0.02±0.02	0.02±0.02	2.02±0.18 [a]
C. citratus + C. medica						
Mix 1	18.90±2.74	1.52±0.15 [a]	0.06±0.04	0.20±0.05	0.02±0.02	1.80±0.27 [a]
Mix 2	15.40±3.66	1.40±0.24 [a]	0.06±0.04	0.06±0.06	0.02±0.02	1.54±0.36 [a]
Mix 3	13.40±2.41	1.18±0.15 [a]	0.08±0.03	0.06±0.02	0.02±0.02	1.34±0.23 [a]

AECC: aqueous extract of *C. citratus*; AECM: aqueous extract of *C. medica*; Mix: AECC + AECM; PI: prophase index; mI: metaphase index; AI: anaphase index; TI: telophase index; MI: mitotic index. Results are expressed as mean ± standard error of the mean (S.E.M.), n = 10; [a] $p < 0.001$ compared to distilled water.

Following exposure to AECC, aberration index (AI) went from 00 ± 00 in the control group to 1.70 ± 0.96, 2.46±1.28, and 3.30 ± 1.19 at the three concentrations tested, respectively. Similarly, AECM increased this parameter which went from 00 ± 00 in the control group to 0.16 ± 0.16, 1.93 ± 0.86, and 1.56 ± 0.65 at the three concentrations tested, respectively. The mixture of the two extracts further increased AI, which went from 00 ± 00 in the control group to 2.00 ± 0.82 (Mix 1), 4.00 ± 0.57 ($p < 0.05$; Mix 2) and 5.33 ± 0.63 ($p < 0.01$; Mix 3) (Table 2 and Figure 2).

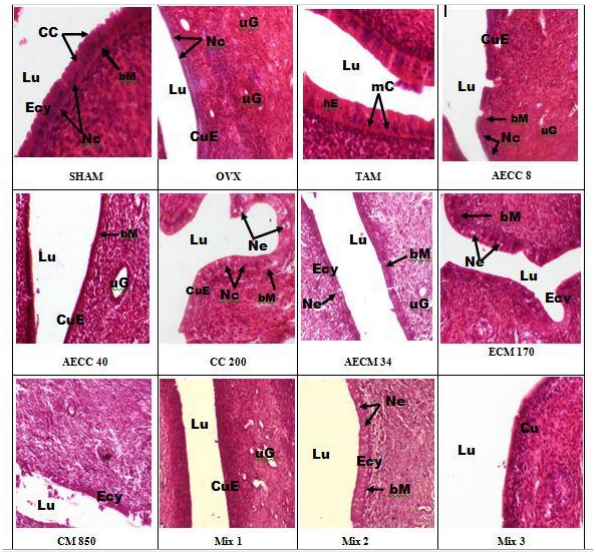


a) normal interphase: presence of homogeneous filaments of chromosomes concentrated in the center of the cell; **b)** normal prophase (arrow): formation of the mitotic spindle and condensation of chromosomes; **c)** normal metaphase (arrow): chromosomes aligned along the midline of the cell; **d)** normal telophase: nucleus formation around each set of daughter chromosomes; **e)** chromosomal adherence (arrow): formation of chromosomal contraction and condensation; **f)** chromosomal break (arrow): presence of a chromosome fragment isolated from batches of paired chromosomes; **g)** chromosomal bridge (arrow): persistent connection between two homologous chromosomes during polar ascension of batches of chromosomes in anaphase; **h)** chromosomal laggard (arrow): chromosomes isolated from different batches of chromosomes during polar ascension **i)** Disturbed Anaphase: unbalanced chromosome distribution, heading them to asymmetric poles of the cell, opposite to what occurs in the normal division cycle; **j)** disorganized metaphase: unbalanced chromosome distribution during metaphase, opposite to what occurs in the normal division cycle.

Figure 2: Chromosomal alterations observed following the microscopic analysis of meristematic cells of *A. cepa*.

3.5. Effects of extracts on the uterus

Figure 3 shows that the uterus of animals in SHAM was bounded by a simple columnar epithelium characterized by epithelial cells taller than

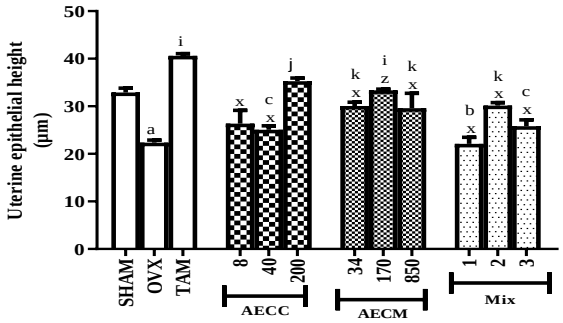


OVX: ovariectomized animals; TAM: tamoxifen; AECC: aqueous extract of *C. citratus*; AECM: aqueous extract of *C. medica*; Mix: AECC + AECM; Lu: lumen; CC: columnar cells; CuE: cubic epithelium; Ecy: columnar epithelium; hE: hyperplastic epithelium; bM: basement membrane; Nc: cell nuclei; uG: Uterine gland; mC: Mitotic cells; Ne: Necrosis

Figure 3: Microphotographs (H&E, 400x) of the uterus of experimental rats. SHAM: non-ovariectomized animals,.

they are wide and a single layer of cell nuclei. A simple cubic epithelium was observed in OVX, the epithelial cells being as tall as they are wide but whose height was much lower than that of the epithelial cells of SHAM. Tamoxifen induced hyperplasia of the uterine epithelium which presents multiple cell layers identifiable by multiple layers of cell nuclei. The co-administration of tamoxifen with AECC or AECM maintained a single layer of cell nuclei characterizing simple cubic/columnar uterine epithelia. The antihyperplastic effect of AECC and AECM was associated with cell necrosis, as shown in certain images in animals receiving extracts of the aforementioned plants.

Figure 4 shows that ovariectomy decreased uterine epithelial height by 32.06% ($p < 0.001$), compared to the SHAM group. Tamoxifen increased uterine epithelial height by 81.62% ($p < 0.001$), compared to OVX. AECC inhibited the tamoxifen effect as it decreased uterine epithelial height by 35.12% ($p < 0.001$), 38.29% ($p < 0.01$), and 13.20% at the three doses tested, respectively, compared to TAM. AECM also opposed the tamoxifen effect as it decreased uterine epithelial height by 26% ($p < 0.001$), 17.09% ($p < 0.05$) and 27.09% ($p < 0.001$) at the three doses tested, respectively, compared to TAM. Similarly, the co-administration of tamoxifen with AECC and AECM reduced this parameter by 45.56% ($p < 0.001$), 25.78% ($p < 0.001$), and 36.47% ($p < 0.001$) respectively, compared to TAM.

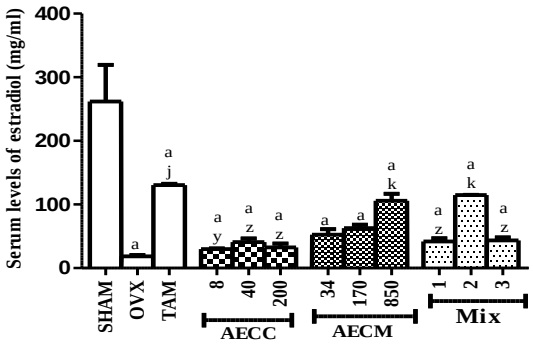


OVX: ovariectomized animals; TAM: tamoxifen; AECC: aqueous extract of *C. citratus*; AECM: aqueous extract of the fruits of *C. medica*; Mix I: AECC + AECM. Results are presented as the mean ± S.E.M. n = 6; a $p < 0.001$; b $p < 0.01$ and c $p < 0.05$ compared to SHAM; i $p < 0.001$; j $p < 0.01$ and k $p < 0.05$ compared to OVX; x $p < 0.001$ and z $p < 0.05$; compared to TAM group

Figure 4: Effects of treatments on uterine epithelial height.
SHAM: non-ovariectomized animals,

3.6. Effects of extracts on serum levels of estradiol

Serum estradiol levels decreased by 92.95% ($p < 0.001$) in OVX, compared to SHAM. Tamoxifen increased this parameter by 607.09%



OVX: ovariectomized animals; TAM: tamoxifen; AECC: aqueous extract of *C. citratus*; AECM: aqueous extract of the fruits of *C. medica*; Mix: AECC + AECM. Results are presented as the mean ± S.E.M. n = 6; [a] $p < 0.001$ compared to SHAM; [j] $p < 0.01$ and [k] $p < 0.05$ compared to OVX; [y] $p < 0.01$ and [z] $p < 0.05$; compared to TAM group. SHAM: non-ovariectomized animals,

Figure 5: Effects of treatments on serum estradiol levels.

(< 0.001), compared to OVX. AECC inhibited the tamoxifen effect as it reduced serum estradiol levels by 77% ($p < 0.01$), 68% ($p < 0.05$), and

75% ($p < 0.05$) at the doses tested, respectively. Similarly, AECM opposed the tamoxifen effect as it reduced serum estradiol levels by 59.85%, 51.75%, and 18.84% respectively, compared to TAM. The co-administration of tamoxifen with AECC and AECM also reduced serum estradiol levels by 68% ($p < 0.05$), 12.03%, and 66.63% ($p < 0.05$), in the three test groups, respectively (Figure 5).

3.7. Effects of extracts on uterine oxidative stress

Uterine levels of MDA in OVX were comparable to those in SHAM. Tamoxifen increased this parameter by 29.16%, compared to OVX. AECC decreased this parameter by 14.51%, 11.29%, and 9.67% at the doses tested, respectively, compared to TAM. AECM also reduced this parameter by 8.06%, 26.53%, and 9.67% at the doses tested, respectively, compared to TAM. The co-administration of tamoxifen with AECC and AECM reduced this parameter by 33.87% (Mix 1), compared to TAM (Table 3).

Tamoxifen reduced by 7.18% the activity of GSH, as compared to OVX. AECC inhibited the tamoxifen effect as it increased this parameter by 7.37%, 9.86%, and 29.76% at the doses tested, respectively, compared to TAM. Similarly, AECM opposed the tamoxifen effect as it increased GSH activity by 32.46% (34 mg/kg) and 8.57% (170 mg/kg). The co-administration of tamoxifen with AECC and AECM further increased GSH activity by 25.62% (Mix 1), 44.62% ($p < 0.01$; Mix 2), and 30.04% (Mix 3), compared to TAM (Table 3).

Tamoxifen decreased SOD activity by 8.76%, as compared to OVX. AECC reversed this effect by increasing this parameter by 48.24% (200 mg/kg; $p < 0.001$), compared to TAM. AECM also increased ($p < 0.05$) SOD activity by 40.19%, 38.94%, and 32.79% at the doses tested, respectively, compared to TAM. The co-administration of tamoxifen with AECC and AECM further increased SOD activity by 31.64% (Mix 1), 92.25% (Mix 2; $p < 0.001$), and 110.98% (Mix 3; $p < 0.001$), compared to TAM (Table 3).

Table 2: Chromosomal and phase alterations in meristematic cells of *A. cepa* exposed to aqueous extracts of *C. citratus* and *C. medica*

Groups	Chromosomal alterations				Phase alterations		Chromosomal aberration (%)
	CA	CB	CBr	CL	DA	DM	
Control	00±00	00±00	00±00	00±00	00±00	00±00	00±00
C. citratus							
AECC 0.25	00±00	4.00±0.94^{b}	0.60±0.24	0.20±0.20	5.40±1.28^{c}	00±00	1.70±0.96
AECC 0.5	00±00	00±00	0.20±0.31	6.00±1.41^{a}	6.80±1.39^{a}	00±00	2.46±1.28
AECC 1	00±00	5.2±0.94^{a}	1.80±0.24	4.80±0.20^{a}	7.40±1.28^{a}	0.60±0.24	3.30±1.19
C. medica							
AECM 0.25	00±00	00±00	00±00	00±00	1.00±00	00±00	0.16±0.16
AECM 0.5	00±00	1.80±0.58	6.00±1.37^{a}	0.60±0.24	1.20±0.20	2.00±0.44^{b}	1.93±0.86
AECM 1	00±00	4.00±0.70^{b}	0.20±0.20	0.60±0.24	2.80±0.20	1.80±0.37^{c}	1.56±0.65
C. citratus* + *C. medica							
Mix 1	2.00±0.77^{b}	1.40±0.50	6.00±1.14^{a}	0.60±0.24	1.40±0.50	0.60±0.24	2.00±0.82
Mix 2	5.40±0.74^{a}	2.20±0.37	5.20±1.15^{a}	2.40±0.24	4.80±0.96^{c}	4.00±0.70^{a}	4.00±0.57^{c}
Mix 3	2.80±0.20^{a}	7.40±0.74^{a}	4.80±0.96^{b}	5.20±1.06^{a}	6.40±1.28^{b}	5.40±0.50^{a}	5.33±0.63^{b}

AECC: aqueous extract of *C. citratus*; AECM: aqueous extract of *C. medica*; Mix: AECC + AECM; CA: Chromosome adherence; CB: Chromosomal bridge; CBr: Chromosomal breaks; CL: chromosome laggard; DA: Disturbed Anaphase; DM: disorganized metaphase. The results are expressed as mean ± standard error of mean (S.E.M.), n = 10; $^{a}p < 0.001$, $^{b}p < 0.01$, $^{c}p < 0.05$ compared to distilled water.

Table 3: Effects of treatments on some uterine parameters of oxidative stress

Groups	MDA (µM/1000 mg of tissue)	GSH (µM/1000 mg of tissue)	SOD (UI/ml/mg of tissue)
SHAM	0,46± 0,04	11,43 ± 0,13	29,92 ± 0,13
OVX	0,48± 0,03	11,69 ± 0,46	32,42 ± 1,54
TAM	0,62± 0,00	10,85 ± 0,22	29,58 ± 0,01
AECC8	0,53± 0,004	11,65 ± 0,49	25,08 ± 1,67
AECC40	0,55± 0,04	11,92 ± 0,41	30,33 ± 0,71
AECC200	0,56± 0,009	14,08 ± 0,40	43,86 ± 0,40^{akx}
AECM34	0,57 ± 0,019	14,37 ± 1,18	41,47 ± 1,40^{by}
AECM170	0,49 ± 0,06	11,78 ± 0,20	41,10 ± 2,10^{cy}
AECM850	0,56 ± 0,049	11,01 ± 0,93	39,28 ± 3,13
Mix 1	0,41± 0,03	13,63 ± 1,30	38,94 ± 1,73
Mix 2	0,88± 0,11^{cky}	15,73 ± 1,86^{cjy}	56,87 ± 3,30^{aix}
Mix 3	0,63± 0,08	14,11 ± 0,40	62,41±3,33^{aix}

SHAM: non-ovariectomized animals, OVX: ovariectomized animals; TAM: tamoxifen; AECC: aqueous extract of *C. citratus*; AECM: aqueous extract of the fruits of *C. medica*; Mix: AECC + AECM. Results are presented as the mean ± S.E.M. n=6; $^{a}p < 0.001$; $^{b}p < 0.01$ and $^{c}p < 0.05$ compared to SHAM; $^{i}p < 0.001$; $^{j}p < 0.01$ and $^{k}p < 0.05$ compared to OVX; $^{x}p < 0.001$ and $^{y}p < 0.01$ compared to the TAM group.

4. Discussion

The present study investigated the chemical composition, cytogenotoxic and antihyperplastic effects of aqueous extracts of *C. citratus* and *C. medica*.

The result obtained from the quantitative analysis of AECC and AECM revealed that these extracts contain amounts of phenols, flavonoids and tannins, lower than those observed by Makni et al.[26] in Tunisia and Unuigbe et al.[27] in Nigeria. These results suggest that the chemical composition of a plant is influenced by various factors including the place of harvest, the part used, the season, geographical and climatic conditions [28], extraction method and extraction solvents [29].

The antioxidant capacity of the two extracts was evaluated by two different methods to obtain more accurate results since the extracts are composed of multiple components with different chemical structures and characteristics. The results of the anti-free radical activities support the hypothesis of the greater antioxidant power of AECC compared to AECM. Indeed, the concentration of AECC capable of trapping 50% of DPPH was 83.75±0.33 µg/mL while that of AECM was 134.89 ± 0.65 mg/mL. From a pharmacological point of view, the lower the EC50 value of a substance, the higher its pharmacological potential [30]. Moreover, the ferric-reducing antioxidant power of AECC was

higher than that of AECM, confirming the higher antioxidant potential of AECC compared to AECM. This *in vitro* antioxidant capacity of AECC and AECM could be attributed to their chemical composition characterized by the presence of antioxidant compounds, in particular phenols. The higher content of phenols and flavonoids in AECC could explain its greater antioxidant power, compared to AECM.

Exposure of *A. cepa* to different concentrations of both extracts made it possible to assess their cytogenotoxic effects. Results showed that these extracts significantly and dose-dependently reduced the number and growth rate of *A. cepa* roots. Such effects have been attributed to inhibition of cell division mechanisms or a decrease in the number of living cells capable of dividing [31]. This effect was associated with a decrease in the number of dividing meristematic cells (cytotoxic effect) and a decrease in the prophase index. The decrease in the rate of cells in prophase would be due to the alteration of microtubules thus preventing the assembly of chromosomes at the metaphase stage [32]. The blocking of cells in prophase resulted in a reduction in the mitosis index. According to Erhabor and Erhabor.[33], the reduction in the mitosis index is an indicator of the mitodepressant effect of a plant. It also reflects the ability of the latter to block the synthesis of DNA and nucleoproteins in the biological system [34].

An increase in the index of chromosomal and phase aberrations (genotoxic effect) was also observed after exposure of onion bulbs to AECC and AECM. These aberrations were characterized by the presence of chromosomal bridges, trailing chromosomes, agglutinated chromosomes, chromosomal breaks, and disturbed Anaphases. The presence of chromosomal bridges in anaphase could be due to the translocation of unequal chromatic exchange or to the presence of dicentric chromosomes. These bridges cause structural chromosomal mutation [35]. The presence of clumped chromosomes has been attributed to degradation or depolymerization of chromosomal DNA [36], or DNA concentration and stickiness of interchromosomal fibers [37]. Chromosome loss would result from the persistence of bridges at the time of pairing of homologous metaphase chromosomes. This formation of bridges leads to breaks during the polar ascent of chromosomes in anaphase [36]. The accumulation of chromosome loss as well as disorganized anaphases contribute to facilitating inter- and intra-chromosomal interactions, responsible for agglutinations; this, in the absence of DNA self-repair [38]. The increase in the aberration index following exposure of the apices of *A. cepa* to different concentrations of AECC and AECM, could be attributed to the presence of genotoxic and mutagenic chemicals (phenols, flavonoids, and tannins) in these extracts. Indeed, polyphenols, flavonoids, and tannins were found to have genotoxic properties [13].

Based on the chemical composition of the two plants, *C. citratus* was expected to be more active than *C. medica*. The results rather showed the opposite: *C. medica* was much more cytogenotoxic than *C. citratus*. This raises questions about the quality/identity of the constituent elements of the different classes of compounds detected in the two plants and suggests the presence of antimitotic compounds in *C. medica* absent in *C. citratus*. In addition, exposure to the two extracts further reduced the number and growth rate of *A. cepa* roots. This reflects a potentiating synergistic interaction of the two extracts. Moreover, the appearance of chromosomal aberrations shows that AECC and AECM are genotoxic and would therefore be good antiproliferative agents.

The second part of the work was to determine the ability of AECC and AECM to inhibit the proliferative effect of tamoxifen on the uterine epithelium. It mainly observed the presence of a single layer of epithelial cells on the histological sections of the uteri of animals treated with these extracts, the decrease in serum estradiol levels, the decrease in uterine levels of MDA, and the increase in uterine levels of antioxidants. These results indicate that AECC and AECM would have inhibited aromatase activity, resulting in the lowering of serum estradiol levels and the inhibition of signaling pathways activated by this hormone including cell proliferation and oxidative stress [7,39]. However, the oxidative stress pathway appears instead to have been maintained as uterine MDA levels remained comparable to those induced by tamoxifen and therefore higher than those of the OVX group. This suggests a continuous production of reactive oxygen species (ROS). However, ROS are known to activate Akt and in turn, promote cell proliferation and decrease the activity of antioxidant agents [40]. In contrast, our results showed that the antioxidants increased and cell proliferation was inhibited. These results therefore suggest that AECC and AECM, thanks to their chemical composition rich in free radical scavenging compounds, would have trapped the ROS generated by tamoxifen, preventing their inhibitory effect on antioxidants. Moreover, thanks to the cytogenotoxic potential of their chemical constituents, AECC and AECM would themselves have induced cytotoxicity in uterine endometrium, justifying the presence of necrotic figures in the uterine epithelium and high levels of MDA, a marker of the peroxidation of the membrane lipids [41]. The hypothesis of the cytotoxic capacity of AECC and AECM is supported by the results obtained on the meristematic cells of *A. cepa*. These results clearly showed that AECC and AECM opposed cell proliferation by altering the process of mitosis and induced cytotoxicity as it reduced the number of dividing meristematic cells. The elevated uterine levels of MDA in animals receiving AECC and AECM would therefore not be linked to the increase in oxidative stress (or increased production of ROS) in the uterine cells, but rather to direct cytotoxicity of the extracts on these cells. In addition, increased uterine levels of antioxidants are consistent with decreased serum estradiol levels, ROS scavenging, and Akt pathway inhibition. Thus the antihyperplastic mechanism of AECC and AECM includes inhibition of aromatase activity, cytogenotoxicity, and antioxidant activity. Such effects would be due to the flavonoids contained in these extracts. These phytocompounds are known for their ability to inhibit aromatase activity [42,41]. Additionally, *C. citratus* and *C. medica* also contain polyphenols and tannins, classes of compounds with antioxidant capacities [43].

5. Conclusion

In summary, the present study showed that the aqueous extracts of *C. citratus* and *C. medica* are mitodepressant and antiproliferative substances as they inhibited the mitosis of *A. cepa* meristematic cells and tamoxifen-stimulated uterine epithelial cell proliferation. These effects have been attributed to their genotoxic properties. These extracts could be therefore considered as potential alternatives for the treatment of precancerous and cancerous diseases, and using the two extracts together might be more beneficial than using them separately.

Ethics statement

The animal experimentation was conducted in conformity with the European community guidelines (EEC Council Direction 2010/63/EU of 22 September 2010), after approval of the research proposal by the scientific committee of the Department of Animal Biology (University of Dschang, Cameroon) on October 10, 2019.

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