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The genotoxic properties of leaf extracts from three common Congolese medicinal plants, *Cogniauxia Podolaena* Baill. (1839), *Dissotis Rotundifolia* (Sm.) Triana (1872) and *Emilia Coccinea* G. Don (1839) using the Ames test, the in vitro micronucleus test and the comet assay

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A B S T R A C T

Cogniauxia podolaena, *Dissotis rotundifolia*, and *Emilia coccinea* are widely used in the Democratic Republic of Congo to treat various ailments, but also as contraceptives, laxatives, painkillers, and to combat malaria**.** Although commonly used in traditional medicine, no genotoxicity or mutagenicity data exist for these species. This study aims to assess their potential in vitro genotoxic and mutagenic effects. The plant material was collected in Kinshasa and authenticated by botanist Blaise Bikandu at the herbarium of the Faculty of Sciences of the University of Kinshasa. Methanol, ethyl acetate, and water extracts of these species were prepared and subjected to the AMES test, the comet assay, and the micronucleus test. Genotoxicity was assessed using ethyl methanesulfonate (EMS) and benzo (a) pyrene (BaP). Extracts from *C. podolaena* tested positive in the AMES test, particularly on the TA98 strain, suggesting frame-shift mutations. Methanol extracts from all three plants caused dose-dependent DNA damage in the comet assay, while limited genotoxicity was observed for the aqueous extract of *C. podolaena*. Co-genotoxic effects were observed with EMS and BaP. Methanol extracts from all species and aqueous/EtOAc extracts from *C. podolaena* induced chromosomal abnormalities in the micronucleus test. The results suggest that these traditional medicinal plants contain genotoxic agents, warranting further toxicological studies.

Keywords: Genotoxicity, *In vitro* micronucleus test, *In vitro* comet assay, Traditional medicine, Antimutagenicity

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Introduction

The use of *Cogniauxia podolaena* and *Dissotis rotundifolia*, two herbaceous species, and *Emilia coccinea* is widespread in the Democratic Republic of the Congo for their interesting pharmacological properties (Tripathy *et al.*, 2016). For generations, the tissues of these plants have been used in traditional medicine throughout sub-Saharan Africa to treat various health problems (Khezri *et al.*, 2024).

Extracts of the vine *C. podolaena* are traditionally used to relieve persistent constipation. They are also employed in the treatment of ascites, generalized oedema, scrotal elephantiasis and hernias (Bouquet, 1969). In addition, various preparations of *C. podolaena* are used to treat conditions such as fibroids, myomatous cysts (Ngbolua *et al.*, 2016); mammary and pelvic abscesses, pelvic inflammatory disease and post-partum haemorrhage (Njamen *et al.*, 2013). The leaves of these species are also valued in traditional medicine for their anthelmintic properties (Mbatchi *et al.*, 2006; Banzouzi *et al.*, 2008) and are applied in the treatment of Buruli ulcers caused by mycobacterial infections (Bayaga *et al.*, 2017). Furthermore, the contraceptive potency of the aqueous extracts has been documented (Peneme *et al.*, 2015) and the roots of *C. podolaena* have been shown to induce abortion in pregnant women (Makemba Nkounkou *et al.*, 2017). The herbaceous plant *D. rotundifolia* Triana is also used as a contraceptive in traditional medicine. Preparations of the plant material are applied vaginally to promote anti-ovulatory effects (Aja *et al.*, 2015); Kabena *et al.*, 2018). *D. rotundifolia* leaves are crushed and the extracts are digested to treat various ailments such as upset stomach, diarrhea, conjunctivitis and sexually transmitted infections, or as an anthelmintic (Buyel, 2018). *E. coccinea*; syn. *E. sagittata* is considered an edible plant in traditional dishes. This alicament is commonly used in the management of wounds, sinusitis and as a poultice for wounds. In addition, E. coccinea extracts are used as analgesics to relieve pain during childbirth, dysmenorrhea, gonorrhea, postpartum and to treat colic in infants. An aqueous extract of *C. podolaena* demonstrated a median lethal dose (LD_{50}) of 3167 mg/kg in mice, while no lethality was observed with the hydroethanolic extract (Itou *et al.*, 2017). Although acute *in vivo* toxicity appears low, Diatewa *et al.* (2002) reported clear hepatotoxic effects in rats given 400 mg/kg/day of *C. podolaena* leaf extract by oral gavage. Compared with controls, exposed animals showed liver damage characterized by parenchymal degeneration, macrovesicular steatosis and significant elevations in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. No lethal dose was established in an oral study in mice for aqueous extract of *D. rotundifolia*, although doses up to 5 g/kg body weight were tested (Abere *et al.*, 2010). However, Ansah *et al.* (2016) reported liver toxicity in Wistar rats that daily received ethanolic extracts of *D. rotundifolia* up to 250 and 500 mg/kg via the oral route. In contrast, no signs of toxicity were observed in a subacute study in which MeOH extracts of *D. rotundifolia* were orally administered to rats in doses up to 1000 mg/kg bw/day for 14 days (Ansah *et al.*, 2016). Toxicity data on *E. coccinea* are scarce. Acute toxicity was also assessed by Elvis-Offiah *et al.* (2016), but they observed no signs of toxicity 24 hours after the administration of MeOH leaf extract.

Despite the frequent use of *C. podolaena, D. rotundifolia* and *E. coccinea* in traditional and folkloric medicine to treat a variety of diseases and ailments, the plants remain poorly studied in terms of their pharmacological properties, chemical composition, health effects and toxicological profile (Chabour *et al.*, 2022). Most traditional medicinal plants have not been subjected to exhaustive toxicological tests. One important toxicological endpoint for which information needs to be collected is genotoxicity, the induction of damage to the genetic material, as it has been associated with serious adverse human health effects, including cancer and heritable diseases (Djidi *et al.*, 2021). The present study investigated the genotoxic profile of extracts obtained from the three plants. In order to collect genotoxicity data, an *in vitro* battery consisting of the bacterial reverse gene mutation test (also referred to as Ames test) and the *in vitro* micronucleus test is generally performed as a first step (EFSA, 2011). The Ames test detects genetic mutations, while the in vitro micronucleus test detects structural and numerical chromosomal aberrations. Therefore, combining these tests allows for the evaluation of all three genotoxic parameters. Genotoxicity, the induction of genetic damage, is a critical toxicological endpoint,

as it is linked to serious health risks such as cancer and inherited disorders. This study aimed to evaluate the genotoxic potential of extracts derived from the three plant species. To obtain meaningful genotoxicity data, a standard in vitro testing approach was used, following EFSA guidelines (EFSA, 2011). This included the bacterial reverse mutation assay, commonly referred to as the Ames test and the in vitro micronucleus assay. The Ames test is designed to detect point mutations at the gene level, while the micronucleus assay identifies both structural and numerical chromosomal changes. Combined, these two assays offer a robust evaluation of the three key genotoxicity endpoints. However, when studying the genotoxicity of plant extracts, antibacterial properties and/or the presence of histidine may interfere with the Ames test (Verschaeve, 2015).

Materials and Methods

Collection of the plant material

Plant material of the three study organisms was collected at the Monastery of the Assumption in Kindele, Kinshasa, Democratic Republic of the Congo (ϕ -4.420317270192991, λ 15.300300637983499) in September 2018. Herbarium samples of the above-mentioned species were collected in collaboration with the Herbarium of Inera-University of Kinshasa (IUK), Department of Biology, Faculty of Science and Technology, University of Kinshasa. The retrieved plant material was collected at the herbarium and the identity of the plants was confirmed to be *C. podolaena, D. rotundifolia* and *E. coccinea* as outlined in the Supplementary data section 1. Post identification, the plants were then air-dried at room temperature. The previously reported chemical composition of the plants is summarized in the supplementary data.

Preparation of the plant extracts

For each sample, extracts were prepared by extracting 10 g of powder with 100 ml of solvent. Three different types of solvent were used i.e., water, methanol (MeOH) and ethyl acetate (EtOAc). The aqueous extract was made by mixing the water/extract solution for 24 h on a stirring plate. Afterwards, the macerate was filtered twice through Whatman 1 filter paper and the collected extract was lyophilized for 24h. In order to obtain the MeOH & EtOAc extracts, the solvent/extract solutions were macerated for 48h followed by filtration of the macerate through Whatman 1 filter paper. The filtrated extracts were concentrated and dried with a Nevap system using a nitrogen stream at room temperature. Before testing, MeOH extracts were dissolved in DMSO with a stock concentration of 50 mg/ml. Due to limited solubility, the stock concentration of the aqueous extracts was 25 mg/ml DMSO, whereas that of the EtOAc extracts was only 10 mg/ml DMSO.

Cell culture

C3A (HepG2/C3A); Human Hepatocellular cells (C3a) (ATCC, CRL-10741) and Chinese hamster ovary (CHO)-K1 cells (ATCC, CCL-61) were purchased directly from ATCC® (United States). Cells were cultured in Dulbecco's modified Eagle's culture medium (DMEM) containing glutamax (1%), sodium pyruvate (1%), non-essential amino acid (1%), gentamicin (1%), amphotericin B (0.1%) and fetal bovine serum (FBS, 10%) (Marta *et al.*, 2018). Cells were maintained in a humidified atmosphere at 37°C with 5 % CO_2 in 75 cm^2 flasks and passaged every 3-4 days. Cell cultures were regularly inspected for the presence of Mycoplasma. *Salmonella typhimurium* TA 98 and TA 100 were subcultured in Oxoid Nutrient broth and cryopreserved in aliquots of 100 µL before use following the protocol of (Abuwatfa *et al.*, 2024).

Bacterial Reverse Gene Mutation Test (AMES)

To determine the mutagenic potential of the plant extracts, an Ames 98/100 test with *Salmonella typhimurium* strains TA 98 and TA 100 as described by Cross (1996) was executed. Both *Salmonella* strains are deficient in the production of histidine (His) due to induced mutations and rely on exogenous addition of His for their survival. Formation of colony-forming units (CFU) on a growth matrix deficient of His is the result of cells that have undergone reversion to amino acid prototrophy for His (Mortelmans and Zeiger, 2000). Both strains differ as the genotype underlying the TA98 strain contains the *hisD3052* mutation while the TA100 strain contains a hisG26 allele. Resulting from the difference in their genetic makeup, the TA98 strain can be applied to identify frame-shift mutations caused by xenobiotics, whilst the TA100 strain is able to report pointmutations. Briefly, 100 µL of bacterial stock was incubated in 20 mL of Oxoid Nutrient broth for 16 h at 37°C on a

rotary shaker. Of this overnight culture, 0.1 mL was added to 2.0 mL of top agar (containing 0.05 M His/ Biotin) together with 0.1 mL test solution and 0.5 mL phosphate buffer (or 4% rat liver S9mix). To determine mutagenicity, the test solution contained 50 µL of the test sample and 50 µL of solvent controls. The positive control in the test with S9 mix was 1 µg/plate 2-aminanthracene (2AA). In the test without S9 mix, the positive controls were 0.2 µg/plate 4-nitroquinoline oxide (4-NQO) for TA98 and 5 µg/ plate sodium azide (SA) for TA100, respectively. Positivity was reported as CFU counts surpassing values that were double the baseline value of the negative control (Masayuki *et al.*, 2018).

***In vitro* alkaline comet assay**

The *in vitro* alkaline comet assay was performed as described by Cross *et al.* (1996) with some slight modifications. Briefly, C3a cells were seeded into 12-well plates at a density of $2x10^5$ cells/well and treated 24h later with 4 different concentrations of the plant extracts for 24 hours. A top concentration of 500 µg/ml, 250 µg/ml and 100 µg/ml was applied for the MeOH, aqueous and EtOAc extracts, respectively. Ethyl methane sulphonate (EMS; 31 µg/mL) and benzo[a]pyrene (BaP; 2 µg/mL) were used as positive control compounds. After exposure, cells were trypsinized, collected and resuspended in 100 µL ice-cold PBS and kept on ice to prevent further DNA damage and repair. For each control or test condition, 50 µL of the cell suspension was mixed with 600 µL of 0.8% low-melting-point agarose at 37°C and 75 µL cell/LMP mix was spread on pre-coated slides (1% normal-melting-point agarose), resulting in the embedding of single cells in agarose gels. Afterwards, the slides were immersed in ice-cold lysing solution (2.5 M NaCl; 100 mM EDTA; pH=10; 10 mM TRIS; 1% Triton X-100 and 10% DMSO) at 4°C overnight. Slides were incubated in alkaline buffer (0.3 M NaOH, 1 mM EDTA, pH>13) for 40 min to allow the unwinding of the DNA and to convert alkali-labile sites to strand breaks. The samples were then subjected to electrophoresis in the same buffer for 20 min (1 V/cm, 300 mA), resulting in the formation of single-cell comets. Cells were rinsed three times for 5 minutes in neutralizing 0.4 M Tris buffer pH 7.5. Slides were allowed to dry; 10 minutes in ice-cold ethanol and air-dried overnight before staining with a 3x gel red solution. Two slides per condition were used for the semi-automated scoring of 200 comet images for each slide. Pictures of the cells were captured and analyzed automatically with the Metafer™ 4 scanning platform (Meta System, Althlussheim, Germany), consisting of a motorized Zeiss Axiovert 40 fluorescence microscope in combination with Metafer4 software. After visual control of the image quality, at least 50 cells per slide (100 cells per condition) were maintained. The percentage DNA in the tail of the comet was used as a measure of DNA damage and the median value for all comets for each condition was calculated. Treated cells were compared to untreated cells for the detection of a statistically significant increase in DNA damage using the non-parametric Mann-Whitney U test with the GraphPad Prism software version 7.01.

For each extract, the impact of the tested concentrations on cell viability was assessed in parallel in the neutral red uptake (NRU) assay in C3a cells. Briefly, C3a cells were seeded into 96-well plates at a density of $4x10^4$ cells/well and 24h later, cells were exposed to the plant extracts for 24h. Cells were washed with phosphate-buffered saline (PBS) and incubated with 200 µL of 50µg/mL Neutral Red. Three hours later, cells were washed with PBS and the neutral red was extracted from the cells with 200 µL of ethanol/acetic acid solution (50:1). After homogenization on a well-plate shaker, the optical density of the extracted neutral red, proportional to the number of viable cells, was detected at 540 nm in a plate reader (Beckman Coulter). The Measured OD 540 nm of unexposed cells was set to 100% viability, from which the cell viability of the exposed cells with the extracts could be calculated.

For the MeOH extracts of the three samples, the anti/co-genotoxic effect was also investigated in the *in vitro* comet assay. To this extent, the extracts were tested as described above, but in the presence of a clearly genotoxic and not too cytotoxic concentration of the direct mutagen EMS (31 µg/mL) and the pro-mutagen BaP (2 µg/mL). Median %DNA in the tail from extract-treated cells together with mutagen was compared to the median % DNA in the tail from mutagen-only-treated cells in order to evaluate significant differences in DNA damage (antigenotoxic or co-mutagenic effects). NRU, as described above, was tested in parallel to evaluate cell viability,

expressed as % viability of the combined effects of mutagen and extract on the cells compared to untreated cells.

In vitro micronucleus test

The *in vitro* micronucleus test was carried out following the OECD guideline 487, with some slight modifications (OECD, 2016). In brief, CHO-K1 cells were seeded at a density of 2.0 × 10^5 cells/mL (with S9) or 1.0 × 10^5 cell/mL (without S9) in a 6-well plate and exposed to four concentrations of the plant extract in the absence (24h) or presence (3h) of the exogenous metabolic activation system S9 (a mixture of aroclor induced rat liver S9 fraction and NADPH cofactor). A top concentration of 500 µg/mL, 250 µg/mL and 100 µg/mL was applied for the MeOH, aqueous and EtOAc extracts, respectively. Methyl methane sulphonate (MMS; 15 µg/mL) in the absence and benzo (a) pyrene (BaP; 25 µg/mL) in the presence of S9 were used as positive controls. After exposure, cells were washed and incubated for 21h with cytochalasin b (CytB; 3µg/mL) to block cytokinesis, resulting in the formation of bi-nucleated cells. After collection and hypotonic treatment with 0.075 mM potassium chloride, cells were fixed three times with a freshly prepared ice-cold CH_3COOH 1:3 MeOH solutions. Few drops of the cell suspension were equally distributed on pre-cleaned slides and cells were stained with 50 µL of Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; 1.5 µg/mL) after overnight drying. Slides were evaluated for the presence of micronucleated binuclear cells with the semi-automated Metafer™ micronuclei scanning and scoring platform (Meta System, Althlussheim, Germany), consisting of a motorized Zeiss Axiovert 40 fluorescence microscope in combination with Metafer4 software. For each test condition, 2 slides were analyzed for the scoring of 1000 binucleated cells per slide, resulting in at least 2000 evaluated cells. The % MN (micronuclei) was calculated for each condition. Results were summarized and statistically analyzed with GraphPad Prism version 7.01 in order to evaluate whether there was a significant increase in % MN of each extract concentration compared to the negative control (Fisher's Exact test $p<0.05$) and if a concentration-dependent genotoxic effect was induced by the extract (chi-square test $p<0.05$).

To evaluate cytotoxicity, one slide for each test condition was stained with acridine orange (AO; 33.3 µg/mL). The number of mono-, bi- and poly-nucleated cells from 500 AO-stained cells was counted manually and the cell proliferation was evaluated using the cytokinesis block proliferation index (CBPI), calculated as:

$$CBPI = \frac{(1 \times mononucleated + 2 \times binucleated + 3 \times polynucleated)}{Total\ cells}$$

Cytostasis percentage and Relative survival percentage as a measure of cytotoxicity were calculated for each test condition by comparing CBPI from exposed to unexposed cells (negative control):

$$Cytostasis\ \% = 100 - (100 \times \frac{(CBPI_{test} - 1)}{(CBPI_{neg} - 1)})$$

$$Relative\ survival\ \% = 100 - Cytostasis\ \%$$

Results

MeOH and EtOAc achieved higher yields compared to Aq. extraction

For each of the three samples, the weight of the dried extract and the recovery yield obtained with the different solvents are summarized in Table 1. Aqueous Extraction with water resulted in the lowest recovery yield. The recovery yields with MeOH and EtOAc were in the same range, with the exception of *Cogniauxia podolaena,* which showed a lower recovery yield with MeOH (6.6%), compared to EtOAc (10.8%).

Table 1. Recovery yield for the different extracts.

	Methanol (MeOH)		Aqueous (Aq.)		Ethyl acetate (EtOAc)	
	Weight of the dried extract (g)	Recovery yield (%)	Weight of the dried extract (g)	Recovery yield (%)	Weight of the dried extract (g)	Recovery yield (%)
C. podolaena	3.311	6.6	0.689	6.0	1.083	
D. rotundifolia	1.164	10.2	0.587	6.0	1.103	
E. coccinea	1.598	12.8	0.824	7.0	1.184	

C. podolaena extracts test positive in the AMES screening by causing frame-shift mutations

Aq., MeOH and EtOAC *C. podolaena*, MeOH *D. rotundifolia* and MeOH *E. coccinea* extracts were selected for investigation by AMES. Compared to the negative control, none of the five extracts selected induced an increase in the number of cells that had undergone reversion to amino acid prototrophy in the *S. Typhimurium* TA100 strain either in the absence or in the presence of S9 metabolic fraction. In contrast, all extracts of *C. podolaena* (MEOH, EtOAc and Aq.) were mutagenic in the *S. Typhimurium* TA98 strain, both in the absence and in the presence of S9 metabolic fraction, increasing CFU reflecting an increase of revertants as shown in Figure 1. The MeOH extracts of *D. rotundifolia* and *E. coccinea* (ME3) were negative in the TA98 strain. Since the *S. Typhimurium* TA98 strain contains the genotype *hisD3052*, the observed reversion is evidently caused by a frame-shift mutation.

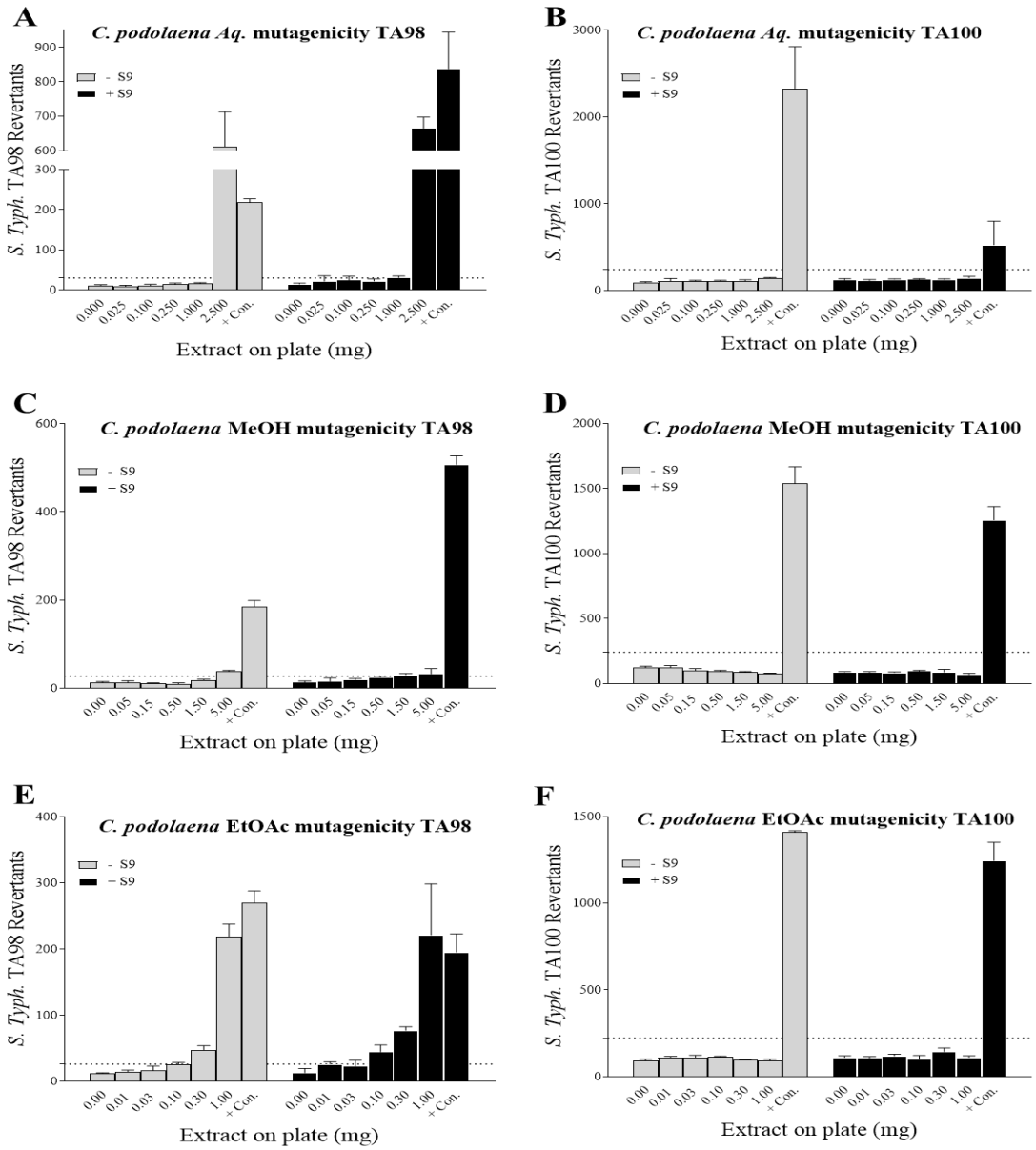

Fig. 1. The mutagenic potential of the *C. podolaena extracts* observed in the AMES test. The Panels A, C and E contain the results obtained with the *S. typhimurium* TA98 to identify frame-shift mutations, while the panels B, D and F contain the results obtained with the *S. typhimurium* TA98 to identify base pair substitution mutations due to exposure to the plant extracts. The dashed line indicates a 2-fold increase over baseline. Data are expressed as the mean of triplicate ± SD. The positive control for samples treated with S9 was 2-aminoanthracene (2-AA; 1.0 µg/plate), for samples without treatment of S9, 4-nitroquinoline-N-oxide (4-NQO; 0.2 µg/plate) was used.

Methanol extracts cause elevated levels of DNA damage observed in the comet assay

When tested in the alkaline comet assay, all three MEOH extracts (*C. podolaena*, *D. rotundifolia* and *E. coccinea)* induced a clear statistically significant and concentration-dependent increase in the % DNA in the tail (Fig. 2). A genotoxic effect was also observed with the EtOAc extract of *C. podolaena,* but only at the highest concentration. Due to solubility issues, the extract could not be tested at concentrations higher than 100 µg/ml. However, some cytotoxicity (i.e., 29%) was already observed at 100 µg/mL in the NRU assay (results not shown). The EtOAc extracts of the other two samples *D. rotundifolia* and *E. coccinea* and all three aqueous extracts were not genotoxic in the *in vitro* comet assay. For these extracts, no or limited cytotoxicity was observed at the highest concentrations tested. Due to the limited solubility, these extracts could not be tested at higher concentrations.

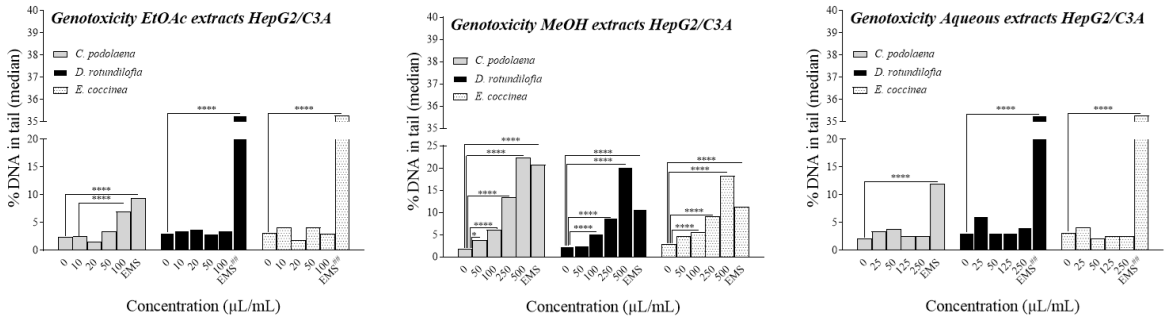

Fig. 2. Genotoxicity results in the *in vitro* alkaline comet assay for the extracts of the three samples obtained with three different solvents i.e., ethanol, methanol and water. */****statistically significant difference compared to negative control (2% DMSO) with p<0.05 or p<0.0001; BaP: benzo (a) pyrene (2 µg/mL); EMS: ethyl methanesulfonate 31 µg/mL; #BaP tested at a concentration of 1 µg/mL; ## EMS tested at a concentration of 62 µg/mL.

Co-genotoxicity with ethyl methanesulfonate and benzo (a) pyrene was observed for the MeOH extracts

The genotoxic effect of the three MeOH extracts was also investigated in the presence of the direct mutagen EMS (31 µg/mL) and the pro-mutagen BaP (2 µg/mL) (Fig. 3). With EMS, co-genotoxicity was observed for all three MeOH extracts, as with each of the extracts, there was a clear statistically significant and concentration-dependent increase in the % DNA in the tail induced by EMS compared to treatment with EMS only. With BaP, more variable results were obtained. Similar to EMS, the MeOH extract of *C. podolaena* induced a clear concentration-dependent co-genotoxic effect with BaP. For the MeOH extract of *E. coccinea*, a co-genotoxic effect with BaP was only present at the highest concentration of the extract. In contrast, an anti-genotoxic effect with BaP was observed with the MeOH extract *D. rotundifolia*. No cytotoxicity was observed for any of the test conditions in the NRU assay (results not shown).

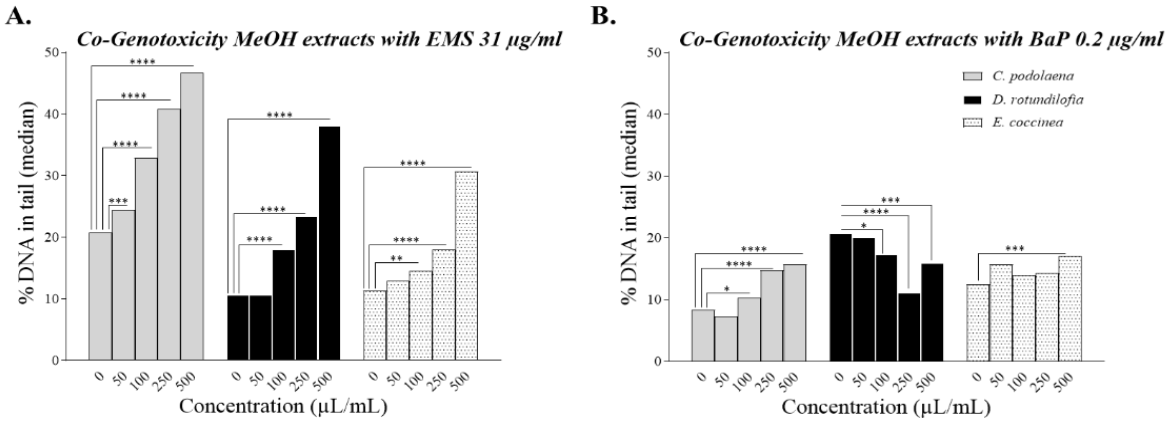

Fig. 3. Anti/Co-genotoxicity results against ethyl methanesulfonate (EMS) (A) and benzo (a) pyrene (B) for three MeOH plant extracts in the *in vitro* alkaline comet assay. Statistically significant difference compared to EMS only or BaP only (*-****p<0.05-0.0001).

The MeOH extracts of all three plants and the EtOAc extracts of C. podolaena cause chromosomal aberrations in the micronucleus assay, both in the presence and absence of S9 metabolic fraction

In the absence of S9 metabolic fraction, all three MeOH extracts induced a concentration-dependent statistically significant increase in the number of micronuclei in binucleated cells (Fig. 4A, C & E). The genotoxic effect was most pronounced with the MeOH extract of *D. rotundifolia*. An increase in the frequency of micronuclei in binucleated cells was also observed with the EtOAc extract of *C. podolaena*. However, like for the MeOH extract of *E. coccinea*, the effect was only observed at the highest concentration tested. No effect on the number of micronuclei in binucleated cells was observed with the EtOAc extracts of the other two samples. The aqueous extract from *C. podolaena* induced a slight but statistically significant increase in the micronucleus frequency in binucleated cells at several concentrations tested. As the effect was limited and not really concentration-dependent, the biological relevance of this effect is considered to be limited. Also, with the aqueous extracts of the other two samples, no effect was observed in the *in vitro* micronucleus test in the absence of S9 metabolic fraction.

In the presence of S9 metabolic fraction, the effect of the plant extracts on the number of micronuclei in binucleated cells was less clear (Fig. 4B, D & F). All three MeOH extracts induced a statistically significant increase in the number of micronuclei in binucleated cells at one or more of the tested concentrations. For both the ethanolic extract of *C. podolaena* and *E. coccinea*, there was a slight but statistically significant and concentration-dependent effect. No effect on the number of micronuclei in binucleated cells was observed with the EtOAc extract of *D. rotundifolia* and with the aqueous extracts of the three plant extracts.

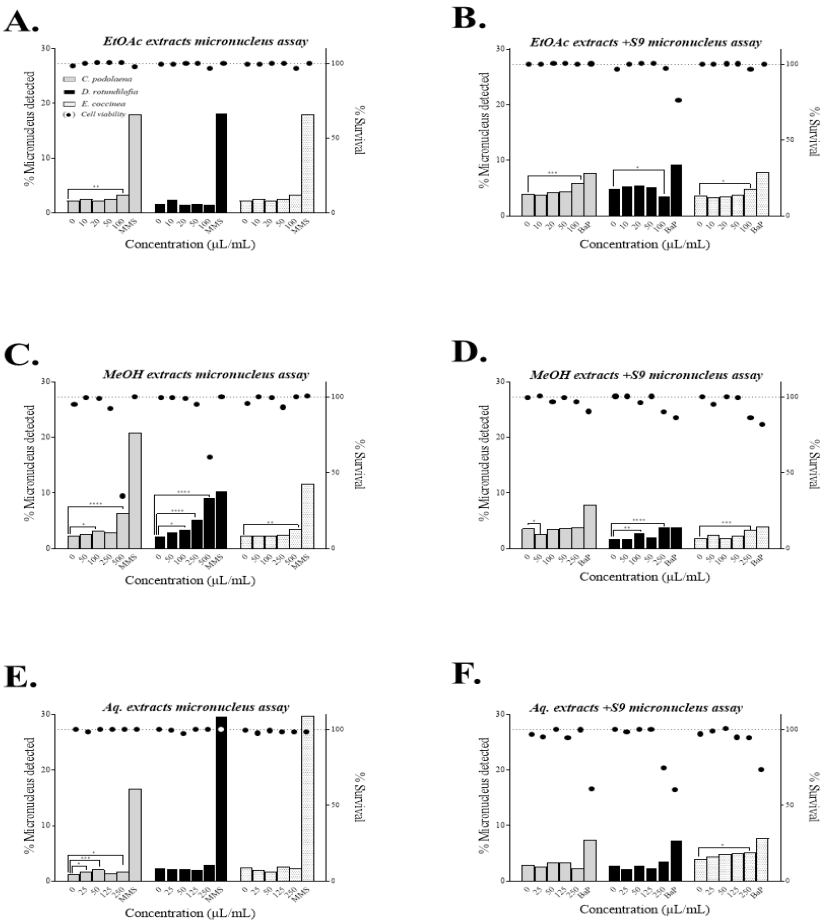

Fig. 4. Results of the *in vitro* micronucleus test in the absence (Panel A, C & E) and presence of S9 metabolic fraction (Panel B, D & F). A-B; EtOAc, Ethyl acetate extracts of *C. podolaena*, *D. rotundifolia* and *E. coccinea*. C-D; MeOH, Methanol extracts. E-F; *Aq.*, *Aqueous* extracts. */****statistically significant difference compared to negative control (0 µg/mL) with $p<0.05$ or $p<0.0001$; MMS: Methyl methanesulfonate (15 µg/mL). BaP: benzo (a) pyrene (25 µg/mL). Cell viability was assessed by the cytokinesis-block proliferation index (CBPI).

Discussion

This study evaluated the in vitro genotoxic effects of methanol, ethanol, and aqueous extracts from the leaves of three different plants with reported medicinal effects: *C. podolaena, D. rotundifolia,* and *E. coccinea*. In the AMES test, only the MeOH and EtOAc extracts of *C. podolaena* tested positive in the *S. typhimurium* TA98 strain, but no mutagenicity could be detected in the TA100 strain. This indicates that the reversion to His prototrophy promoted by the extracts is facilitated by a frame-shift mutation rather than a point mutation. Furthermore, it could be argued that the increase in CFU is due to the presence of residual His in the plant extracts. However, this hypothesis is not supported by the absence of effects on strain TA100 after exposure to plant extracts. These results are in line with those obtained by Eweka *et al.*, (2017). *D. rotundifolia* an increase in chromosomal aberrations was observed after CHO-K1 cells were exposed to MeOH extracts from the leaves of the three selected plants. The addition of the S9 metabolic fraction partially attenuated the effect, as higher concentrations were required to achieve the same effect. Exposure of CHO-K1 cells to the aqueous extract of *C. podolaena* also increased micronuclei. These results are in line with those obtained by Focho *et al.* (2009).

Finally, co-genotoxicity with the known mutagens EMS and BaP was observed in the in vitro comet assay, as exposure to a mixture of EMS or BaP and MeOH extract from the leaves of all three plants resulted in a higher proportion of fragmented genomic DNA than the sum of exposure to EMS or BaP and MeOH extract from the plant taken separately. Anti-genotoxicity was observed with MeOH extracts from *D. rotundifolia*, probably due to interference with Bap metabolism by S9.

Conclusion

Indications of in vitro genotoxicity were observed for leaf extracts of *C. podolaena*, *D. rotundifolia* and *E. coccinea*. Importantly, genotoxicity was observed in the AMES test, the comet assay and the micronucleus test. All three plants are well-established plants in traditional medicine with frequent use to cure a plethora of ailments. Although further study is evidently needed, caution should be taken with the consumption or topical application of preparations containing *C. podolaena, D. rotundifolia* and *E. coccinea* for medicinal purposes. The study underlines the need for a more in-depth toxicological and genotoxicological investigation of plants used in traditional and folkloric medicine. Ultimately plants pose an extremely valuable and inexhaustible source for natural products with therapeutic properties.

Author contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

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