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IN VITRO AND *IN VIVO* GENOTOXICITY ASSESSMENT OF TOTAL ALKALOIDS OF *AGERATUM CONYZOIDES L.* LEAVES (ASTERACEAE) BY ALKALINE COMET ASSAY

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Keywords:

Ageratum conyzoides, Alkaline Comet Assay, genotoxicity, PC3 cells, mice PBMC.

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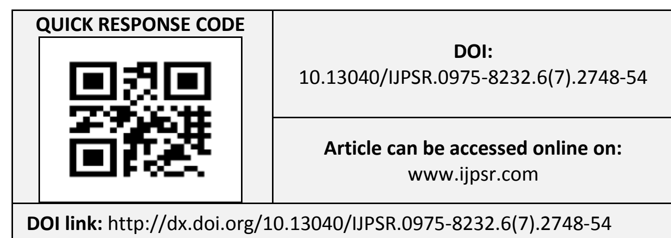
ABSTRACT: In African traditional medicine, *Ageratum conyzoides* L. has been used as purgative, febrifuge, anti-ulcer and wound dressing. *A. conyzoides* contains pyrrolizidine alkaloids, a class of hepatotoxic, genotoxic and carcinogenic phytochemicals. This study aims to evaluate the genotoxicity of total alkaloids of *A. conyzoides* leaves by the alkaline-comet assay.The comet assay was done on human prostate cancer (PC3) cells and on mice PBMC cells. Our results have demonstrated that, *in vitro*, the hydroalcoholic extract (200 µg/mL) and total alkaloids (200 µg/mL) of *A. conyzoides*, which have shown the high cytotoxicity on PC3 cells with MTT assay, increased significantly ($p<0.001$) DNA damage index (DI) and DNA damage frequency (DF) of PC3 cells. The non-alkaloids and the methanolic extract of *A. conyzoides* had no effect on DI and DF. *In vivo*, the administration of total alkaloids of *A. conyzoides* at 750 mg/kg for 3 days increased significantly ($p<0.01$) the DI and the DF of mice PBMC cells. Thus, in this study, by *in vitro* and *in vivo* comet assay test, we have shown that total alkaloids of *A. conyzoides* at 750 mg/kg can induce genotoxicity.

INTRODUCTION: Pyrrolizidine Alkaloids (PAs) are a class of hepatotoxic, carcinogenic, genotoxic, teratogenic and sometimes pneumotoxic phytochemicals. Several reports in the literature prove the fact that PA-containing plants are hazardous for livestock. For a long time, it has also been well established that humans can be affected by toxic PAs [1-6].



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About 3% of the world flowering plants contain toxic pyrrolizidine alkaloids. They are found in more than twelve higher plant families, among which three families, Boraginaceae, Compositae (Asteraceae), and Legumionsae (Fabaceae), contain most toxic pyrrolizidine alkaloids [6-10].

Ageratum conyzoides L. is an annual herbaceous plant belongs to Asteraceae family. It's known to possess a broad spectrum of medicinal, pharmacological and therapeutic properties[11-12]. In African traditional medicine, *A. conyzoides* has been used as purgative, febrifuge, anti-ulcer and wound dressing [13-14]. Traditional communities in India, use this plant as a bactericide, antidysenteric,

and antilithic [15-16]. In Togo, *A. conyzoides* is reported to treat fever, measles and snake bites[13]. In addition to its popular use for skin diseases, wound healing, diarrhoea, pain associated with navel in children, in Nigeria it was reported in the treatment of HIV/AIDS [12, 15]. Our previous studies have shown that the limit dose of 5,000 mg/kg did not cause any mortality or any signs of acute toxicity in rats tested. In 28 days subchronic test, the result did not show any treatment–related abnormalities except the relative weight of the liver where there was a significant increase [13]. This increase in the relative weight of the liver can have a toxic origin or not [17-19]. The results of 90 days subchronic toxicity study have shown that *A. conyzoides* at 500 and 1000 mg/kg can induce liver, kidney and haematological disorders [20].

Some pyrrolizidine alkaloids have been identified in *A. conyzoides*[7-10] and few reports have shown the genotoxicity of lycopsamine and echinatine, two pyrrolizidine alkaloids present in this plant [7, 15]. Because of the presence of these pyrrolizidine alkaloids in *A. conyzoides* L., the ratio benefit/risk should be carefully examined before any therapeutic use of this plant [21-25]. The presence of two genotoxic alkaloids in *A. conyzoides* does not mean that the whole plant or total alkaloids of this plant will be also genotoxic, an antagonist effect of some compounds is possible. Therefore, we aim in this study to evaluate the genotoxicity of total alkaloids of *A. conyzoides* leaves Alkaline Comet Assay.

MATERIELS ET METHODS:

Chemicals:

Comet lysis buffer (2.5M sodium chloride, 100 mM EDTA (pH 8.0), 10 mM Tris–HCl (pH 8.0), 10% DMSO, 1% Triton X-100). Comet alkali solution (0.3 M sodium hydroxide, 1 mM EDTA). Comet neutralizing buffer (0.4 M Tris–HCl, pH 7.5). Cryopreservation medium (50% foetal calf serum [FCS], 10% DMSO, 40% Dulbecco's modified Eagle's medium [RPMI]). Resuscitation medium (50% FCS, 10% dextrose, and 40% RPMI). Culture medium for PC3 cells (RPMI, 10% FCS, 50U/ml penicillin, 50U/ml streptomycin, 2mM L-glutamine). All chemicals were purchased from Sigma Chemicals Co., Hymedia (India).

Collection and extraction of plant materials:

A. conyzoides was collected from Djagble (Togo) in July 2013. It was identified by Prof Kouami Kokou from the Botany department of University of Lome (Togo) and a voucher specimen was kept in the herbarium of the Laboratory of Botany and Plant Ecology (Faculty of Science/University of Lome) under the reference N° 10553 of Tchala. The samples were dried during 2 weeks under air-conditioned room (18°C).

A. conyzoides fractions extraction:

About 500 g of *A. conyzoides* were extracted with 80% methanol by maceration. The combined extracts were evaporated to dryness under reduced pressure giving methanolic fraction (MeOH). The methanolic fraction was then dissolved in 0.5 M HCl and filtered. The filtrate was washed with chloroform until the washings were colourless. The chloroform extract gave a negative test with Dragendorff reagent, indicating the absence of alkaloidal salts in the organic solvent. This chloroformic fraction was concentrated to produce the non-alkaloids fraction (NA). The acidic extract was made alkaline with concentrated ammonia and extracted with chloroform. The latter chloroform extract were combined, dried (Na_2SO_4), filtered and concentrated to dryness under reduced pressure to yield alkaloid fraction 1.

The residual ammonical solution was made acid and stirred with zinc dust for 2 h, centrifuged and decanted. The supernatant was then made alkaline with concentrated ammonia and again extracted with chloroform to give N-oxime fraction. The two alkaloidal fractions were combined for the biological evaluations[26]. For the hydroalcoholic extract, dried powder of *A. conyzoides* was soaked in ethanol-water (90-10: v/v) for 72 h with manual discontinue agitation. The solution was filtered and evaporated (yield: 12.34).

Determination of constitute by TLC and colorimetric reactions:

The alkaloid extracts and the other extracts (50 mg) were dissolved in methanol and made up to known volumes (5ml). After, 5µl of each solution were spotted on a TLC silica gel 60 F254 pre-coated aluminium sheet. Toluene-ethyl acetate 7:3, after pre-saturation in the twin trough chamber were used as mobile phase. After the run, plates are dried

and sprayed by Dragendorff and Erlich reagent. For the Dragendorff, the plates after spraying were allowed at room temperature for 10-15 min. After spraying the Erlich reagent (2 g dimethylamino benzaldehyde, 50 mL of ethanol 95%, 50 mL concentrated HCl) the plates were dried at 85°C for 5 min. The presence of alkaloids is indicated by an orange color with the Dragendorff reagent and red magenta color with the Ehrlich reagent [26].

Cell culture: PC3 (Human prostate cancer cell line) was obtained from NCCS Pune. It was maintained in Roswell Park Memorial Institute (RPMI) supplemented with, amphotericin (3 g/µl), gentamycin (400µg/ml), streptomycin (250 µg/ml), penicillin (250 units/ml) ; 10% fetal bovine serum (FBS) in a carbon dioxide incubator at 5% CO_2.

MTT assay: Approximately 1000 cells/well were seeded in 96 well plate using culture medium. After 24hrs, the new medium with extracts in the concentrations ranging from 0.1µg/ml to 100µg/ml were added at respective wells and kept incubation for two doubling times. After incubation the fresh medium was changed again for all groups and 10µl of MTT (5 mg/ml) was added and the plates were incubated for an additional 4h. The medium was discarded and the formazan blue, which was formed in the cells, was dissolved with 100µl of DMSO. The optical density was measured at 570nm. Survived cells in treated wells were expressed as percentage of control wells. The IC_{50} (50% viability inhibitory effect) was determined and expressed in µg/mL[27].

Comet assay *in vitro*:

The principle of the comet assay is that smaller DNA molecules migrate faster in an electric field than larger molecules. The treated cells are encapsulated in gel and lysed by alkali, which also denatures the DNA. Subsequent electrophoresis causes migration of the DNA. While the undamaged DNA appears as a 'head', fragmented DNA move faster, giving the characteristic appearance of a comet tail.

The comet assay was performed as described by Singh *et al.,*[28]. Cells were placed in a 6 multi-well plate: 1 ml at 5×10^6cells/ml in RPMI. The cells were allowed to attach for 24 h at 37°C. After 24 h, the medium was replaced by RPMI containing 100 µM H_2O_2 or *A. conyzoides* extracts at different concentrations and duplicate cultures were incubated at 37°C for 30 min. At the end of incubation, the cells were harvested and used in *comet assay* analysis of DNA damage. The cells were checked for their viability before the start and after the end of the reaction using Trypan Blue Exclusion Test [29]. The viability of the cells was found to be greater than 95%.

Partly frosted microscope slides were pre-coated with 1% normal melting-point agarose (NMA) and allowed to dry for 24 h at 37°C. An aliquot of 10µl of PC3 cells were mixed with 75µL of 0.5% LMPA and covered immediately with a cover slip. Once set, the cover slip was removed and a final layer of 75µl 0.5% LMPA was applied to the existing gel to form an agarose sandwich, with the cells in the middle layer. The final cover slip was removed once the gel had set. For each PC3 sample, two slides were prepared. At this point the lights were switched off and the remainder of the assay was performed using indirect light.

After removal of the cover slip, the slides were placed in lysis buffer [2.5 mol/L NaCl, 100 mmol/L Na_2EDTA, 10 mmol/L Tris (pH 10)] with freshly added 1% Triton X-100 and 10% DMSO for at least 1 hour at 4°C. Subsequently, slides were placed in the electrophoresis chamber and incubated with electrophoresis alkaline buffer [300 mmol/L NaOH, 1 mmol/L Na_2EDTA (pH >13)] for at least 20 minutes at 4°C to allow for DNA unwinding and the expression of alkali-labile DNA damage as strand breaks. Electrophoresis was performed for 30 minutes at 25 V and 300 mA.

The slides were then washed three times, for 5 min. each; with neutralization buffer [0.4 mol/L Tris (pH 7.5)]. Finally, slides were stained with 50µL of ethidium bromide (2 mg/mL), covered with a cover slip and observed at 40 magnifications in an Olympus fluorescence microscope. Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analysed for each test substance concentration. Cells were scored visually into five classes, according to tail size (from undamaged – 0, to maximally damaged – 4); and a damage index (DI) and damage frequency (DF)

value was assigned to each comet according to its class. Visual scoring of comets is a valid evaluation method determined by international guidelines and recommendations for the comet assayb[30]. DF, which is the proportion (%) of cells presenting tails after electrophoresis, was also considered in this study [31, 32].

Comet assay *in vivo*:
Albino Mice (weighing 25-30 g) were obtained from PSG Institute of Medical Sciences Research animal house. The animals were kept in plastic cages in an experimental room under controlled conditions of temperature (22 ± 2°C), humidity (55 ± 10%), 12 h light/dark cycles and ad libitum access to food and water. They were randomized at the beginning of the experiment. The study design was approved by Ethics Committee of PSG Institute of Medical Sciences Research under the reference number: 208/2013/IAEC. The experiments were undertaken in accordance with the ethical principles of OECD.

The mice were divided into 4 groups, with 5 animals per group. Cyclophosphamide (CP) (PSG hospital pharmacy) was used to induce DNA damage. Just before use, the CP was diluted in distilled water and administered by intraperitoneal (ip) injection (200 mg/kg b.w).

The mice in group 1 received only distilled water (10 mL/kg b.w. per day by gavage) for 3 days prior to treatment with distilled water by intraperitoneal (i.p.) injection. Although they have also received distilled water (10 mL/kg b.w. per day by gavage) for 3 days, those in group 2 was treated with CP (200 mg/kg b.w.) on the 3rd day. Individuals in groups 3 and 4 have received total alkaloids of *A. conyzoides* prepared in two different doses, 150 mg/kg (group 3) and 750 mg/kg (group 4), for 3 days prior to a distilled water i.p. injection on the 3rd day. Doses of 150 and 750 mg/kg are close to therapeutic doses we have used in our previous study and those found in several articles[11].

The mice were killed by ether anesthesia, 24 h after CP and extracts treatment, for evaluation of comet assay. Samples of peripheral blood were collected from mouse retro-orbital sinus. After the viability test, PBMC was isolated by using Histopaque 1077. Then cells were mixed with 0.5% LMPA and the comet assay was done like above.

Statistical analysis: The results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed by one way analyse of variance (ANOVA) with Tukey test to evaluated significant differences between groups. Values of $p< 0.05$ were considered significant. All statistical analysis were carried out using the Instat Statistical package (Graph Pad software, Inc. USA).

RESULTS & DISCUSSION:

***A.conyzoides* fractions cytotoxicity:** The total alkaloids yield was 0.085% and the yield of N-oxime was 0.0088%. Because of the small quantity of N-oxime, we combined it with total alkaloids. The Draggendorf reagent have shown that, all our extract, even the non-alkaloid fraction, contain some amount of alkaloids. With the Ehrlich reagent we have shown that the alkaloids present in our fraction have an indole in their structure. Tryptamine was used to confirm the positive reactivity of the Ehrlich reagent. *A. conyzoides* fractions IC_{50} and fractions alkaloids level are presented in the **Table 1**. Alkaloids and hydroalcoholic extract are more cytotoxic than methanolic and non-alkaloid extract.

All the extracts except the non-alkaloids are rich in pyrrolizidine alkaloids. *A. conyzoides* is used in traditional medicine of many places in the word. It's used in the treatment of a lot of diseases. Like most of the plants, *A. conyzoides* it seems to be non-toxic[13]. In this study, our results have shown that *A. conyzoides* contain pyrrolizidine alkaloids or at least indole alkaloids. Lycopsamine and echinatine, two pyrrolizidine alkaloids have been identified in *A. Conyzoides* [7-10]. Pyrrolizidine alkaloids are secondary plant metabolites. Due to their potentially harmful effects (carcinogenic effect) on health they are undesirable in food and feed [7-8].

***In vitro* effect of *A. conyzoides* extracts on the DNA fragmentation:** The hydroalcoholic extract (200µg/mL), total alkaloids (200µg/mL) and hydrogen peroxide (100µM) increased significantly ($p<0.001$) the DI and the DF of PC3 cells (**Fig. 1**

and **Table 2**). The increase of DI and DF was more important with total alkaloids and the positive control (hydrogen peroxide) than with the hydroalcoholic extract.

***In vitro* effect of total alkaloids of *A. conyzoides* on the DNA fragmentation:**
Total alkaloids increased significantly ($p<0.001$) at 200 µg/mL the DI and the DF of PC3 cells. Hydrogen peroxide also increased significantly ($p<0.001$) the DI and the DF of PC3 cells (**Table 3**).

***In vivo* effect of total alkaloids of *A. conyzoides* on the DNA fragmentation:**

In vivo, only the dose of 750 mg/kg after 3 days administration increased significantly ($p<0.01$) the DI and the DF of mice PBMC. A single I.P. injection of cyclophosphamide increased also significantly ($p<0.001$) the DI and the DF of mice PBMC (**Table 4**).

TABLE 1: *A. CONYZOIDES* EXTRACT IC50 ON PC3 CELLS AND THEIR CONTAINING IN PYRROLIZIDINE ALKALOIDS

Extract	Dragendorff	Ehrlich	IC_{50} µg/ml
Hydroalcoholic extract	++	++	85.38
Methanolic extract	+	+	>100
Total alkaloids	+++	+++	46.67
Non alkaloids extract	+	+	>100

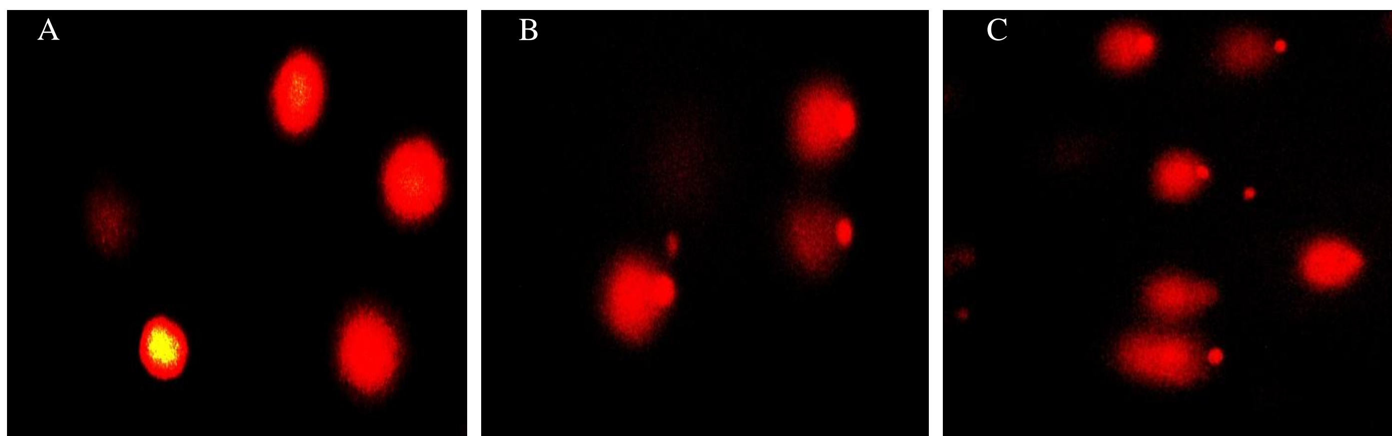

FIG.1: PHOTOMICROGRAPHS OF DAMAGED DNA OBSERVED AFTER THE TREATMENT OF PC3 CELLS BY *A. CONYZOIDES* EXTRACT (A) CONTROL; (B) HYDROALCOHOLIC EXTRACT 200 µg/mL (C) HYDROGEN PEROXIDE (100 µM)

TABLE 2: EFFECT OF *A. CONYZOIDES* EXTRACTS ON THE DNA FRAGMENTATION *IN VITRO*

Extract	% of cellular inhibition	DI	DF (%)
Control	1	9 ± 9	5 ± 3
Hydroalcoholic extract (200 µg/mL)	1	75 ± 13 *	80 ± 11**
Methanolic extract (200 µg/mL)	0	7 ± 7	6 ± 4
Total alkaloids (200 µg/mL)	4	136 ± 20***	90 ± 9***
Non alkaloids extract (200 µg/mL)	0	20 ± 11	11 ± 6
Hydrogen peroxide (100 µM)	3	267 ± 21***	98 ± 7***

Data are means ± SEM. * significantly different as compared to control with $p<0.05$. ** Significantly different as compared to control with $p<0.01$. *** significantly different as compared to control with $p<0.001$/one way ANOVA with Tukey test. DI: damage index; DF: damage frequency.

TABLE 3: EFFECT OF TOTAL ALKALOIDS FROM *A. CONYZOIDES* ON THE DNA FRAGMENTATION *IN VITRO*

Extract	% of cellular inhibition	DI	DF (%)
Control	0	9 ± 9	9 ± 8
Total alkaloids (50 µg/mL)	0	27 ± 14	22 ± 8
Total alkaloids (200 µg/mL)	0.4	136 ± 20***	90 ± 9***
Hydrogen peroxide (100 µM)	0.3	263 ± 20***	96 ± 7***

Data are means ± SEM. *** significantly different as compared to control with $p<0.001$/one way ANOVA with Tukey test. DI: damage index; DF: damage frequency.

TABLE 4: EFFECT OF TOTAL ALKALOIDS OF *A. CONYZOIDES* ON THE DNA FRAGMENTATION *IN VIVO*.

Extract	% of cellulaire inhibition	DI	DF (%)
Control	0	0.00 ± 0.00	0 ± 0
Total alkaloids (150 mg/kg)	0	0.1 ± 0.01	0.1 ± 0.01
Total alkaloids (750 mg/kg)	0	241 ± 20***	75 ± 8***
Cyclophosphamide (200 mg/kg)	0	235 ± 26***	86 ± 9***

Data are means ± SEM. ***significantly different as compared to control with p<0.001/one way ANOVA with Tukey test. DI: damage index; DF: damage frequency.

The use of plants in therapies is a worldwide phenomenon. Currently, drugs derived from plants are being investigated for the possible presence of cytotoxic, mutagenic and genotoxic substances, as well as other biological activities. The detection and evaluation of the cytotoxic, mutagenic and carcinogenic effects of plant compounds is crucial for minimizing the possible risks of these agents, especially when they are part of a long-term treatment[31].

In vitro, on PC3 cells, total alkaloids of *A. conyzoides* and its hydroalcoholic extract increased the DNA damage seen by the comet assay. The Single-Cell Gel Electrophoresis (SCGE), or the comet assay, is a highly sensitive method for assessing DNA damage formation and repair, both at clinically relevant and low doses. Alkaline-comet assaying was undertaken as described by Singh *et al.,*[28]. The comet assay combines the simplicity of biochemical techniques for detecting DNA single strand breaks (frank strand breaks and incomplete excision repair sites), alkali-labile sites and crosslinking with the single cell approach typical of cytogenetic assays. The advantages of the SCGE technique include: (1) the collection of data at the level of the individual cell, allowing for more robust types of statistical analyses; (2) the need for small numbers of cells per sample (<10,000); (3) its sensitivity for detecting DNA damage; and (4) that virtually any eukaryotic cell population is amenable to analysis.

The non-alkaloids and the methanolic extract have not increased the DNA damage of PC3 cells. With Dragendorff and Ehrlich reagent, we have seen that this non-alkaloids fraction contain a very less amount of alkaloids. This result is close to the cytotoxicity of our extracts. With the MTT assay, non-alkaloids and methanolic extracts of *A. conyzoides* have shown an IC50 on PC3 cells over 100 mg/mL when the IC50 of total alkaloids and hydroalcoholic extract was respectively 46.67µg/mL and 85.38µg/mL. The cytotoxicity of hydroalcoholic and total alkaloids of *A. conyzoides* after 30 minutes of incubation on PC3 (shown with trypan blue) was very less. At the same moment, the DNA damage induced by these two extracts was significative (p<0.001). Then the DNA damage took place before the cytotoxicity of *A. conyzoides* hydroalcoholic and total alkaloids in cancer cells lines.

In order to see the genotoxicity of our extract on normal cells lines, we have done the comet assay on mice PBMC. The administration of *A. conyzoides* (750 mg/kg) to mice for 3 days induced significant blood DNA damage. Total alkaloids of *A. conyzoides* have induced blood genotoxicity *in vivo*. Few reports have shown the genotoxicity of *A. conyzoides* [7, 15, 33]. Even if some compound of *A. conyzoides* such as lycopsamine and echinatine have been shown to be genotoxic, it does not means that the whole plant will be genotoxic. We have shown, in this study, by *in vitro* and *in vivo* alkaline comet assay, that *A. conyzoides* total alkaloids at high concentration can induce genotoxicity. Therefore the use of this plant, in traditional medicine, must be controlled.

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